The Role of SOX9 in Phenotype Switching in Melanoma

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1. Summary

Malignant melanoma is the most aggressive skin cancer in the world. Currently, melanoma has undergone the highest increase in incidence worldwide and is the sixth most common cancer. Although melanoma only comprises 4% of all skin cancers, it is responsible for 80% of all skin cancer deaths. When patients are diagnosed with stage I or II melanoma, they are often cured after surgical excision of the primary lesion. However, if the melanoma is diagnosed with stage IV melanoma where distant metastasis has occurred, the one year survival rate is 62%. Thus more effective therapies are needed to successfully treat melanoma. Understanding the molecular mechanisms that cause melanoma progression will be crucial for developing novel drug targets. Melanoma is a heterogeneous cancer, which poses a substantial challenge for therapy as multiple subpopulations need to be targeted for effective treatment. Our group has proposed the phenotype switching model for melanoma heterogeneity where melanoma cells can reversibly switch between states of proliferation and invasion. The two distinct melanoma cell phenotypes are named proliferative and invasive and defined by specific gene expression signatures and *in vitro* characteristics.

The aims of my PhD thesis were to validate the proliferative and invasive gene signatures in a larger set of melanoma cell cultures, to investigate the role of SOX10 and SOX9 in melanoma progression and to investigate the DNA methylation landscape of melanoma in the context of phenotype switching.

Firstly, we found the proliferative and invasive gene signatures were present in the majority of melanoma cell cultures used by many research groups worldwide and the proliferation and invasive capacities predicted by the gene signature of some of these melanoma cell cultures were functionally validated by *in vitro* experiments.

Secondly, we found SOX9 and SOX10 to be antagonistic regulators of each other in melanoma development *in vitro* and *in vivo*. The *Tyr::Nras*^{Q61K} mouse is hyperpigmented and has multiple melanocytic naevi. SOX10 haploinsuffiency in the *Tyr::Nras*^{Q61K} mouse results in normal pigmentation and no naevi. SOX9 deletion in a *Tyr::Nras*^{Q61K} *SOX10*$^{fl/-}$ mouse restores hyperpigmentation and naevi formation. Overexpression of SOX9 in melanoma cells downregulates SOX10 and knockdown of SOX10 upregulates SOX9. In melanoma patients, high SOX10 expression correlates with poor survival. Taken together, the loss of SOX10 upregulates SOX9, consequently inhibiting melanoma tumor formation.

Thirdly, characterization of the DNA methylation landscape of the proliferative and invasive phenotypes revealed SOX9 as a target of DNA hypermethylation in the proliferative phenotype. Overexpression of SOX9 can reduces proliferation but induces invasion in melanoma cells and increase metastasis in mice. In melanoma patients, high SOX9 expression correlates with poor patient prognosis. Taken together, although SOX9 reduces the growth of the tumor, it allows the tumor cells to become migratory which lead to poor patient survival.

In summary, phenotype switching is an important mechanism for melanoma development. Genes that define the phenotypes like SOX10 and SOX9 have functional roles in driving disease progression and could be potential targets for future melanoma therapy.

2. Zusammenfassung

Das maligne Melanom ist der aggressivste Hautkrebs weltweit. Aktuell ist das Melanom die Krebsart mit den meisten Neuerkrankungen und damit das sechst-häufigste Krebsleiden. Obwohl nur etwa 4% aller Hautkrebsfälle als Melanom diagnostiziert werden, sind 80% aller hautkrebs-assoziierten Todesfälle Melanompatienten. Solange Patienten mit Stadium I oder II ihrer Erkrankung diagnostiziert werden, kann eine Heilung durch eine vollständige operative Entfernung des Tumors erfolgen. Befindet sich die Krebserkrankung aber schon im Stadium IV, indem sich bereits Fernmetastasen gebildet haben, beträgt die Überlebenschance im ersten Jahr gerade mal 62%. Aus diesem Grund ist es dringend nötig, effektivere Therapiemöglichkeiten für Patienten mit metastasierten Melanomen zu schaffen. Hierbei ist das Verständnis der molekularen Mechanismen der Melanommetastasierung essentiell um neue Therapieansätze zu schaffen.

Die momentane Herausforderung für einen effizienten Therapieansatz ist die Heterogenität verschiedener Zellpopulationen innerhalb eines Tumors zu eliminieren. Unsere Arbeitsgruppe hat hierzu das „phenotype-switching“ Modell eingeführt, welches vorschlägt, dass Melanomzellen reversibel zwischen zwei Zuständen von Proliferation oder Invasion wechseln können, was zu der beobachteten Heterogenität beiträgt. Ob eine Tumorzelle proliferativ oder invasiv ist wird durch bestimmte Genexperessionsmuster und das *in vitro* Verhalten der Melanomzellen bestimmt.

Die Ziele der vorliegenden Doktorarbeit sind die Validierung der proliferativen und invasiven Genexperessionsmuster in einer grösseren Anzahl Melanomzelllinien, das Erforschen der Rolle

der Proteinfaktoren SOX10 und SOX9 bei der Melanomprogression, sowie das Erforschen der DNA-Methylierungsmuster im Kontext des „phenotype-switching“ Modells.

Erstens fanden wir die proliferativen, sowie invasiven Genexpressionsmuster in dem grössten Teil der getesteten Melanom-Zellkulturen aus verschiedenen Forschungslabors weltweit und konnten diese Genexpressionsmuster mit dem *in vitro* Verhalten von Proliferativen, sowie invasiven Zellkulturen funktionell korrelieren.

Zweitens, wurde gefunden, dass SOX9 und SOX10 antagonistische Regulatoren während der Entwicklung des Melanoms sind, sowohl *in vitro* als auch *in vivo*. Die gentechnisch veränderten Mäuse *Tyr::Nras*^{Q61K} sind hyperpigmentiert und entwickeln multiple melanozytäre Läsionen. SOX10 Haploinsuffizienz in *Tyr::Nras*^{Q61K} gentechnisch veränderten Mäusen resultiert in normaler Pigmentation und keinerlei Entwicklung von melanozytären Läsionen. Die Deletion von SOX9 in *Tyr::Nras*^{Q61K} *SOX10*$^{fl/-}$ gentechnisch veränderten Mäusen hingegen zeigt wieder den Ursprünglichen Phänotyp mit Hyperpigmentation und der Entwicklung von melanozytären Läsionen. Die Überexpression von SOX9 in Melanom-Zellkulturen verringert die Expression von SOX10 und der Verlust von SOX10 erhöht die Expression von SOX9. In Expressionsdaten von Melanompatienten korreliert eine hohe SOX10 Expression in den Tumoren mit geringeren Überlebenschancen. Zusammenfassend kann gesagt werden, dass der Verlust von SOX10 Proteinexpression dazu führt, dass SOX9 Proteine eine erhöhte Expression erreichen und somit die Tumorbildung inhibieren.

Drittens, zeigte die Charakterisierung von DNA-Methylierungsmustern der proliferativen und invasiven Phänotypen , dass der DNA Abschnitt für das SOX9 Gen hypermethyliert ist, jedoch ausschliesslich in der DNA von proliferativen Melanomzellen. Die Überexpression von SOX9 reduziert die Proliferationsrate, aber Erhöht auch die Invasivität der Melanomzellen und die Metastasenformierung in Mäusen. Bei Melanompatienten korreliert hohe SOX9 Expression in den Tumoren mit schlechter Prognose. Obwohl SOX9 die Wachstumsrate von Tumoren verlangsamt, erhöht es auf der anderen Seite die Migration der Krebszellen, und das führt dann zu einer schlechteren Prognose für die Patienten.

Zusammenfassend lässt sich sagen, dass das „phenotype-switching“ Modell ein wichtiger Mechanismus für die Melanomentwicklung ist. Gene, welche die Zellphänotypen prägen, wie die hier beschriebenen Faktoren SOX10 und SOX9, haben funktionelle Aufgaben in der Krankheitsentwicklung und können somit als potentielle Zielmoleküle für zukünftige Melanomtherapien betrachtet werden.

3. Introduction

Melanoma epidemiology

Cutaneous melanoma arises from transformed melanocytes which reside in the basal layer of the epidermis and are derived from the neural crest during development (1). It is the most fatal skin cancer, although it only comprises about 5-7% of skin malignancies it causes up to 75% of skin cancer related deaths due to its capacity to rapidly metastasize (2). The incidence of cutaneous melanoma is rising faster than any other solid tumor. In Switzerland, the incidence of melanoma is the third highest in the world (20.3 cases per 100, 000 people) after Australia (34.9 cases per 100,000 people) and New Zealand (35.8 cases per 100,000 people) (3). Although the 5 year survival is quite high for primary melanomas at (i.e. 98%), once the melanoma has metastasized the 5 year survival rate is only 16.1% (4).

Melanoma staging

Melanoma is staged according to the guidelines set by the American Joint Committee on Cancer (5). The TNM staging system is used to determine the size of the tumor (T) as determined by Breslow's thickness and ulceration, the presence or absence of regional lymphatic metastases with the number of lymph nodes involved (N), and the presence or absence of distant metastases (M). This score provides the TNM staging for the melanoma as detailed in Table 1.

Table 1: The 2009 staging system for cutaneous melanoma according to the AJCC.

Classification	**Thickness (mm)**	**Ulceration Status/Mitoses**
T		
Tis	NA	NA
T1	≤1.00	a: Without ulceration and mitosis <1/mm2 b: With ulceration or mitoses ≥1/mm2
T2	1.01–2.00	a: Without ulceration b: With ulceration
T3	2.01–4.00	a: Without ulceration b: With ulceration
T4	>4.00	a: Without ulceration

		b: With ulceration
N	No. of Metastatic Nodes	Nodal Metastatic Burden
N0	0	NA
N1	1	a: Micrometastasis* b: Macrometastasis†
N2	2–3	a: Micrometastasis* b: Macrometastasis† c: In transit metastases/satellites without metastatic nodes
N3	4+ metastatic nodes, or matted nodes, or in transit metastases/satellites with metastatic nodes	
M	Site	Serum LDH
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

Abbreviations: NA, not applicable; LDH, lactate dehydrogenase.
*Micrometastases are diagnosed after sentinel lymph node biopsy.
†Macrometastases are defined as clinically detectable nodal metastases confirmed pathologically

Melanoma risk factors

The main environmental factor risk factor is ultraviolet (UV) light exposure, which is causally related to melanoma risk. UV light is composed of UVA and UVB wavelengths. UVA is defined as waves ranging 400 to 315 nm and UVB is defined as waves ranging 315 to 280 nm. UVA are less energetic than UVB and are not known to cause direct DNA damage but can cause indirect DNA damage by free radical and reactive oxygen species generation (6-8). UVB is known to cause C-T or G-A transitions which result in pyrimidine dimers that lead to mutated proteins.

This UVB damage signature can be seen by genome sequencing studies in melanoma (9, 10). These C to T transition occur in many tumor suppressor genes including CDKN2A, PTEN and TP53 (see Molecular Subtypes) and a smaller portion of them occur in oncogenes such as RAC1, STK19, FBXW7, IDH1 and TERT (11). In fact, melanoma is the cancer with the highest mutation rate where C to T transitions predominate in the mutation distribution (12). UVB radiation has been demonstrated to accelerate melanomagenesis and produce UVB damage signature mutations in a BRAF V600E mutant melanoma mouse model (13).

Skin pigmentation is also a known risk factor for developing melanoma. The Fitzpatrick skin type classification defines 6 skin phenotypes based on their response to sun exposure (14). There is an inverse correlation with melanoma risk and skin type from fair skin to pigmented skin (Figure 1)

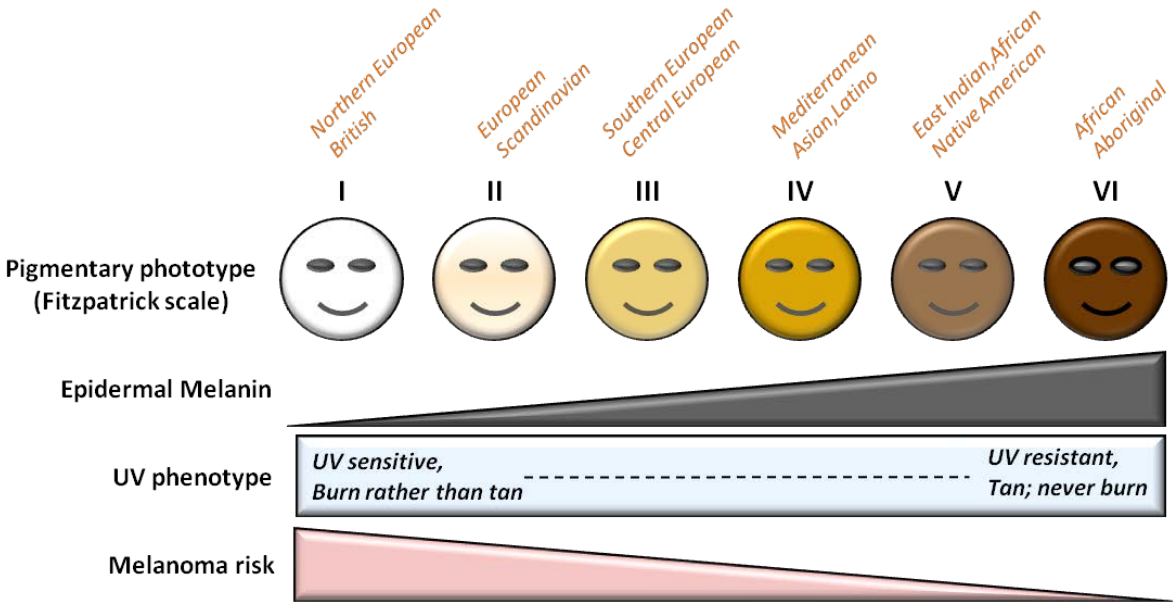

Figure 1 – Melanoma risk vs skin type. Fitzpatrick classification of skin type with correlation to pigmentation, response to UV and melanoma risk. From D'Orazio, JA 2013

It has been estimated that 5-10% of all cutaneous melanomas occurs in families with hereditary melanoma predisposition (15). Point mutations in the CDKN2A locus account for 20-40% of these familial melanoma cases (16-19). The CDKN2A locus encodes for two tumor suppressors, $p14^{ARF}$ and $p16^{INK4A}$, which regulate the p53 and retinoblastoma (RB) pathways respectively. Activating point mutations in cyclin-dependent kinase 4 (CDK4) are also known to

cause familial melanoma, but these are much rarer with only 17 cases reported (20, 21). CDK4 is a gene required for cell cycle progression and activating mutations allow for uninhibited cell growth. Germline mutations in the promoter region of the telomerase reverse transcriptase gene (TERT) were found to be associated with familial melanoma and interestingly somatic mutations in the same region are also found frequently in sporadic melanoma cases (22, 23). These mutations increase the transcription of TERT. And most recently, germline mutations in the gene protection of telomeres 1 (POT1) were identified as increasing susceptibility for familial melanoma (24, 25). POT1 mutations were found in about 4% of familial melanoma cases that lacked CDKN2A and CDK4 mutations.

Mutations in pigmentation genes like melanocortin 1 receptor (MC1R) and microphthalmia-associated transcription factor (MITF) have also been associated with familial melanoma independent of their function in skin pigmentation. MC1R is a G protein-coupled receptor that binds to melanocortins such as melanocyte-stimulating hormone (MSH) and activates MITF for melanin production. Partial loss of function mutations are associated with phototype 1: fair skin, red hair and poor tanning and also associated with increased risk of developing melanoma independent of sun exposure (26, 27). MITF is an essential transcription factor required for activation of many genes which lead to melanocyte differentiation and pigmentation. A point mutation was found to impair normal SUMOylation of MITF leading to increased activation of MITF and increased risk of developing melanoma (28).

Taken together, environmental factors, skin phenotype and genetic factors all contribute to an individual's risk of developing melanoma.

Clinical Subtypes

There are four major clinical subtypes of primary cutaneous melanoma: superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, and acral lentiginous melanoma. These four subtypes are distinguished by their histologic growth pattern, anatomic site, and degree of sun damage. Whether melanoma subtypes influence the overall prognosis remains controversial.

Superficial spreading melanoma accounts for about 70% of cutaneous melanoma and is the most common subtype in adults aged 30-50 years. It is most common on the trunk in men and women and on the legs of women. Irregular asymmetric borders are characteristic.

Histologically, it is characterized by pagetoid scatter of atypical melanocytes within the epidermis.

Nodular melanoma accounts for about 15-30% of cutaneous melanoma and is most commonly seen on the legs and trunk of men and women. Rapid growth occurs over weeks to months and it is responsible for most thick melanomas.

Lentigo maligna melanoma is a pigmented slow growing macule often arising from chronic sun exposed skin. The *in-situ* precursor lesion, lentigo maligna, is usually a greater than 1 cm diameter pigmented macule that has been present for a minimum of 10-15 years. Progression to lentigo maligna melanoma occurs upon dermal invasion and is characterized by the development of raised blue-black nodules.

Acral lentiginous melanoma is the least common clinical subtype in Caucasian individuals and the most common in African, Asian, and Hispanic individuals. It occurs on the palms, soles, and beneath the nail plate. It presents with a flat phase with similar appearances and changes to superficial spreading melanoma.

Melanocytes and melanoma

Melanocytes are pigment producing cells located at the basal layer of the epidermis and are derived from neural crest stem cells (NCSCs) during embryonic development. Initially NCSCs are multipotent and as they migrate throughout the embryo their multipotent potential gradually becomes lineage restricted due to the micro-environmental influences encountered during their journey. They eventually become a specific differentiated cell depending on their final location which includes a plethora of different cell types such as glia, neurons, smooth muscle cells, adipocytes, osteoblasts, chondrocytes, and melanocytes. A number of transcription factors regulate the neural crest migration and differentiation process, one of which is sex-determining region Y (SRY)-box 10 (SOX10). SOX10 is essential for these cells to maintain their multipotency (29). Homozygous deletion of SOX10 in mice leads to embryonic lethality, whereas SOX10 haploinsuffiency causes aganglionosis of the colon and pigmentation defects (30-33).

In the adult body, NCSC subpopulations still exist in location specific stem cell niches and the skin has one of these stem cell compartments which contain a subpopulation of NCSCs called melanocyte stem cells (34-36). Melanocyte stem cells are located in the hair follicle and will

differentiate and produce pigment for the hair during successive rounds of hair growth (37). SOX10 is expressed throughout all stages of the melanocyte lineage (38) and has two essential roles in the melanocytic lineage; firstly, to establish and maintain of the melanocyte stem cell population and secondly, to regulate the production of pigment from melanocytes (39). SOX10 regulates many targets during neural crest development including MITF which is considered the master regulator for normal melanocyte development (40, 41). SOX10 expression has been detected at varying levels in melanoma by immunohistochemistry (42, 43) and high SOX10 expression is correlated with poor prognosis (44, 45). A decrease in SOX10 expression results in increased p21 and p27 expression, inhibitors of cyclin-dependent kinases (CDKs), and subsequently causes cell cycle arrest (46). *In vivo,* SOX10 haploinsufficiency in $Nras^{Q61K}$ and $Grm1^{Tg}$ mice reduce melanoma formation. NRAS and GRM1 activate the mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) signaling pathways, which are important pathways for melanomagenesis (46, 47). From these studies, it seems that not only SOX10 is important for normal melanocyte function but aberrant expression of SOX10 can cause melanoma.

Molecular Subtypes

The mutational landscape of melanoma has been elucidated in several large exome sequencing studies (9-11) and has allowed the identification of many driver mutations and supporting mutations. This led to the molecular characterization of melanoma driven by specific oncogenes in the MAPK pathway.

BRAF is the most common gene mutated in melanoma (35% to 45%) (48). BRAF is one of the kinases in the MAPK kinase cascade. The four main effectors of this pathway are NRAS, BRAS, MEK and ERK. The frequency of the NRAS mutation is also quite high (20-30%) and is mutually exclusive with BRAF. Thus MAPK mutations comprise about 55-75% of the driver mutations in melanoma. The most common mutation in BRAF results in a valine to glutamic acid substitution at codon 600 ($BRAF^{V600E}$) allowing BRAF kinase activity to be constitutively active. The most common mutations in NRAS occur at glutamine 61 ($NRAS^{Q61K}$, 65%) and glycine 12 ($NRAS^{G12V}$, 35%) which prevent efficient hydrolysis of RAS GTP to RAS GDP (49, 50), thus allowing constitutive activity of RAS GTP. Interestingly, the BRAF and NRAS mutations are not UVR induced mutations suggesting a UVR independent mechanism for melanoma initiation. In the remaining population of melanomas that do not have oncogenic BRAF or NRAS mutations,

about 10% have inactivating mutations in neurofibromin (NF1), a negative regulator of Ras. NF1 mutations have been seen in conjunction with BRAF exon 11 activating mutations and in BRAF and NRAS double wild-type melanomas (51, 52). The high frequency of mutations leading to aberrant MAPK activation indicates this signaling cascade to be the main pathway melanoma utilizes for development. Activated NRAS can also signal through the PI3K pathway, which also promotes proliferation and survival.

c-KIT is the receptor tyrosine kinase for stem cell factor (SCF) upstream of NRAS. Mutations in exons 11 and 13 comprise about 85% of all c-KIT mutations (53). Activating mutations in c-KIT or amplification lead to activation of the MAPK and PI3K pathways. c-KIT mutations are most common in mucosal melanomas, acral lentiginous melanomas and melanomas arising from chronically sun damaged skin (54, 55).

Point mutations are not the only genetic aberrations seen in melanoma, copy-number gains and losses have also been extensively characterized. AKT3, a downstream effector in the PI3K pathway, has been shown to be amplified in a fraction of melanomas and functionally leads to the development of melanoma (56). PTEN, a well-known tumor suppressor, is silenced in about 50-60% of melanomas most commonly by focal deletion or allelic loss (9, 57-59). Loss of PTEN leads to constitutive activation of the PI3K pathway and is often associated with the $BRAF^{V600E}$ mutation (60). In contrast to familial melanoma where CDKN2A is inactivated by point mutations, in sporadic melanoma CDKN2A is silenced by allelic loss or focal deletion which occurs in about 20-40% of all melanoma cases (57, 61). MITF, a gene mutated in familial melanoma, also has the same point mutation in sporadic melanoma (28). In addition to the point mutation, amplification of MITF occurs in about 20% of melanoma leading to aberrant MITF activity (62). MITF is also a target of the MAPK pathway, thus highlighting the importance of MITF as central node in melanoma development.

In summary, the molecular landscape of melanoma is very diverse and heterogeneous (Figure 2). Multiple oncogenic driver mutations can initiate melanoma and the diverse array of molecular aberrations that support the driver mutation can create a complex situation for treatment. Although most of the focus in the past decade has been on the MAPK pathway for drug development, the recent surge in whole exome and genome studies have provided many potential therapeutic targets in alternate signaling pathways.

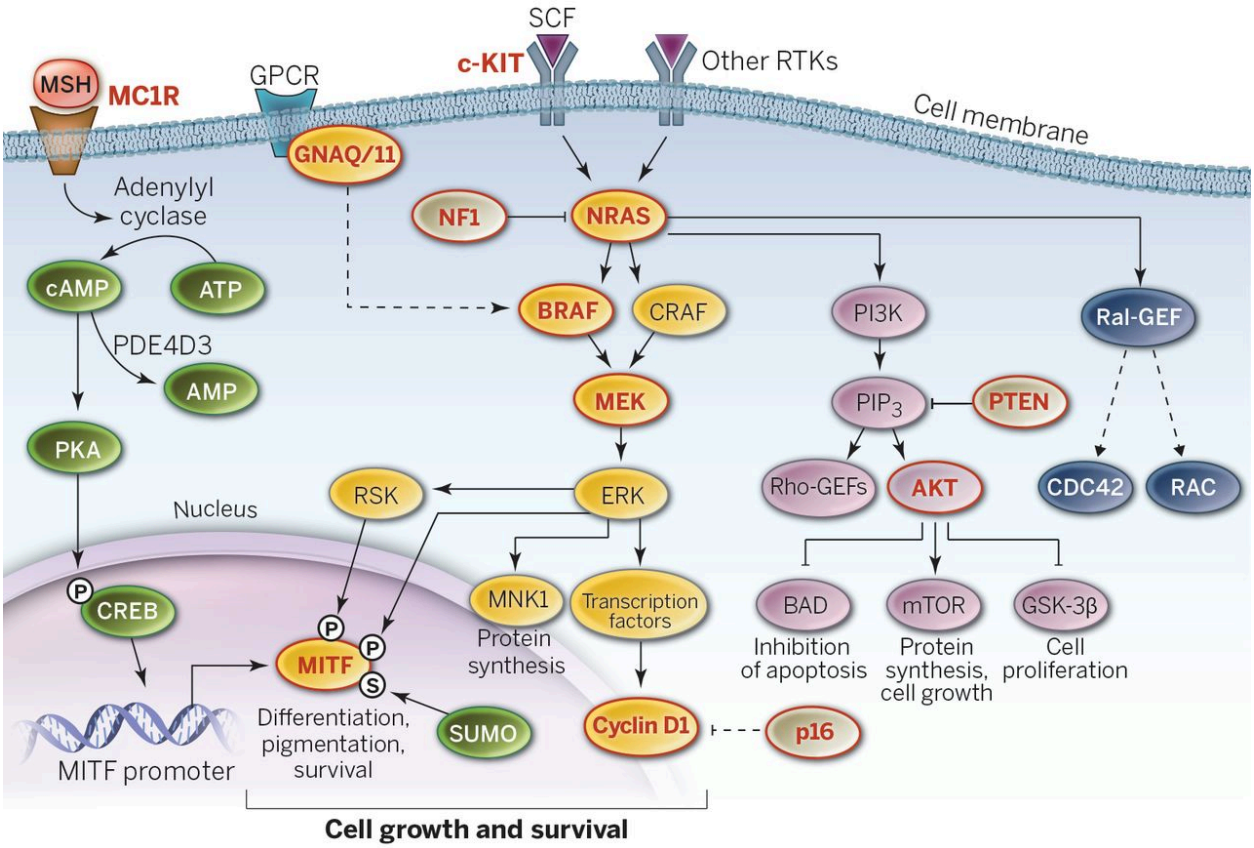

Figure 2. Signaling pathways involved in melanoma development. Genes highlighted in red are known to have genetic aberrations in melanoma. The majority of aberrations are located in the MAPK pathway consisting of c-KIT, NRAS, NF1, BRAF, MEK and ERK which activate MITF among other transcription factors that lead to cell growth and survival. MC1R can activate MITF independent of MAPK signaling. The PI3K pathway complements the MAPK pathway by also inducing proliferation and survival signals . (Lo JA, 2014)

Melanoma therapy

In recent years, several landmark clinical trials for targeted-therapy have boosted the patient survival rate but many patients still relapse after targeted therapy (63). The first targeted therapy to display significant benefit over standard chemotherapy was vermurafenib, a small molecule inhibitor specifically targeting the BRAF V600E mutation. In a phase III trial, the overall response rate of 48% was seen in the vermurafenib group compared to the dacarbazine group, which only had an overall response rate of 5% (64). That led to FDA approval in 2011. Dabrafenib, another $BRAF^{V600E}$ inhibitor with similar results was approved in 2013 (65). Despite the promising results from the clinical trials of these inhibitors, only about half of the patients

with a $BRAF^{V600E}$ mutation benefit from targeted therapy and patients that do benefit initially, inevitably relapse, resulting in a median progression free survival time of 5 to 7 months. Thus, many groups have been investigating the intrinsic and acquired resistance mechanisms to $BRAF^{V600E}$ inhibitor therapy.

Multiple mechanisms of acquired resistance have been elucidated and most involve reactivation of the MAPK pathway. Activation of receptor tyrosine kinases like Insulin-like growth factor IR (IGF-IR) (66), platelet-derived growth factor receptor B (PDGFRB) (67), and epidermal growth factor receptor (EGFR) (68) have all been shown to confer resistance to BRAF inhibitor therapy. The therapy can fail due to BRAF splice variants where a truncated form of $BRAF^{V600E}$ is expressed and can dimerize with full length $BRAF^{V600E}$ leading to activation of MEK (69). Also $BRAF^{V600E}$ amplification was found to confer resistance to $BRAF^{V600E}$ therapy (70). Downstream of BRAF, MEK1 and MEK2 activating mutations have been detected in the resistant tumors (71, 72). Up-regulation of COT1/MAP3K8, an activator of MEK has also been elucidated (73). NRAS mutation and loss of NF1 (67, 74) have also been detected and they activate the PI3K pathway in conjunction to the MAPK pathway. The surrounding microenvironment can release stromal-derived hepatocyte growth factor and activate the MET tyrosine kinase receptor on melanoma cells to signal through the MAPK and PI3K pathways to bypass $BRAF^{V600E}$ inhibitor treatment (75). To add another level of complexity of acquired resistance, intratumoral heterogeneity was observed in some patients; Van Allen et al. reported that multiple independent resistance mechanisms described above were observed within the same resistant tumor biopsy (72). Given the numerous intricate mechanisms that a tumor can employ to become resistant, multiple drug combinations targeting the MAPK and PI3K pathway might be necessary to prevent resistance.

Intrinsic resistance mechanisms to the $BRAF^{V600E}$ inhibitor include aberrant activation of the PI3K pathway, namely AKT3 amplification and loss of PTEN (76, 77), increased CDK4 expression (78), NF1 loss (51, 52, 74), activated MET and SRC signaling (79), and stromal secretion of HGF that activates MET on melanoma cells (75). Recently, phenotype switching has also been shown to be an intrinsic resistance mechanism to $BRAF^{V600E}$ inhibitor therapy (80). Briefly, phenotype switching is a model for melanoma that describes two cell states, one with MITF expression and one with MITF low expression regardless to mutation status. $BRAF^{V600E}$ mutant cells with high MITF were more responsive to inhibitor treatment than to cells with low MITF. These studies suggest that molecular screening for these intrinsic resistance

mechanisms could be beneficial in $BRAF^{V600E}$ patients to determine if they will respond to $BRAF^{V600E}$ inhibitor therapy.

Immunotherapy, which involves the blockade of immune checkpoint inhibition, has also been quite successful in the treatment of melanoma in the past couple of years. T cell activation requires T cell receptor recognition of an antigen presented on the surface on an antigen presenting cell (APC) and a second costimulation step that is mediated by either a stimulatory or inhibitory receptor-ligand pairs known as immune checkpoints. Cytotoxic T lymphocyte antigen-4 (CTLA4) is an inhibitory molecule expressed on T cells and inhibits binding of CD28 on T cells to B7 proteins on APCs thus weakening the costimulation of T cells and CTLA4 is also expressed by melanoma cells (81). Ipilimumab, an anti-CTLA4 antibody, was FDA approved in 2011 from a phase III trial which showed an overall response rate of 11% in the ipilimumab group compared to the dacarbazine group of 5%; although the response was limited, it was quite durable (82). Most recently, pembrolizumab, an anti-PD-1 antibody, was FDA approved in 2014 from a promising phase I study which showed an overall response rate of 52% and less autoimmune toxicities compared to ipilimumab (83, 84). PD-1, like CTLA4, is also an inhibitory receptor which binds to PD-L1 and PD-L2 and melanomas express PD-L1 (85). One of the large benefits of immunotherapy over targeted therapy is all patients regardless of genetic background are eligible for treatment. Resistance mechanisms to immunotherapy are not as well elucidated as targeted therapy, but a few have been explored. Phenotype switching has also been suggested as a resistance mechanism to immunotherapy. Adoptive T cell transfer (ACT) is an immunotherapy that involves selection for cytotoxic T cells that target melanocytic antigens. In an ACT murine model, melanoma cells were seen to switch between a differentiated and a dedifferentiated phenotype in response to T-cell driven inflammatory signaling molecules, like tumor necrosis factor alpha (TNF-α), in a reversible manner inducing resistance to ACT therapy (86). This method would suggest that cytotoxic T cells that target the dedifferentiated phenotype could be beneficial to prevent resistance.

Phenotype Switching

Phenotype switching has been the subject of intense investigation and many groups have contributed to the characterization of the proliferative and invasive phenotypes seen in melanoma (Figure 3). The proliferative phenotype is classified by melanocytic gene expression such as MITF, Melan-A, and SOX10. The invasive phenotype is classified by mesenchymal

gene expression such as Wnt5A, transforming growth factor beta (TGFB) and fibroblast growth factor 2 (FGF2). MITF is known to be the master regulator of melanocyte development and is generally overexpressed in melanoma (87). MITF is essential for melanoma cell proliferation and melanoma cells with low MITF expression have decreased proliferation but increased invasion (88-93). The low MITF expressing cells have many mesenchymal signaling pathways upregulated like the TGFB pathway (94, 95), the sonic hedgehog (SHH) pathway (91), the Brn-2/NOTCH pathway (92, 93), the AXL/NF-kB pathway (80) and the Wnt/B-catenin pathway (96, 97). Both MITF high and MITF low populations give rise to tumors in immunocompromised mice, although the MITF low populations give rise to larger and faster growing tumors suggesting the MITF low population to be more tumorigenic (89). Inhibition of TGFB signaling by SMAD7 overexpression (98) and by a TBRI inhibitor SD-208 (99) reduces melanoma bone metastases. GLI2, a transcription factor activated by sonic hedgehog signaling, inhibits MITF expression and is required for invasion *in vitro* and *in vivo* (95, 100). The transcription factor Brn-2 also inhibits MITF expression and activates NOTCH to induce invasion of melanoma cells (93), and intra-vital imaging of melanoma tumors showed that when melanoma cells become motile, they increase BRN2 expression and when melanoma cells become stationary they decrease BRN2 expression (92). Pinner and colleagues were the first to show *in vivo* evidence for reversible phenotype switching. Low MITF expression was seen to be inversely correlated with NF-kB signaling and the expression of the receptor tyrosine kinase AXL (80). NF-kB activation antagonized MITF expression and increased gene expression of invasion phenotype genes. AXL and NF-kB expression decrease the susceptibility to $BRAF^{V600E}$ inhibitor therapy, suggesting that this MITF low NF-kB high subpopulation have intrinsic resistance. Bittner et al (101) first demonstrated that the gene Melan-A/Melanoma Antigen Recognized by T cells-1 (MART-1) was inversely correlated with Wnt5A expression in a large microarray study for nineteen melanoma samples and that WNT5a distinguished more aggressive tumors from less aggressive tumors. In a follow-up study, Weeraratna et al demonstrated that Wnt5A expression directly correlated with increasing tumor grade and that Wnt5A activated PKC signaling and led to increased invasion (102). Dissanayake et al showed that Wnt5A overexpression or knockdown using siRNA in melanoma cells identified several genes that were suppressed by Wnt5A overexpression, including metastasis suppressors such as Kiss-1 (97). In the same study they also highlighted the inverse relationship between Wnt5A and various tumor-associated antigens of melanocytic lineage, including TYRP-1, DCT, gp100, and MART-1. Wnt5A can down-regulate the expression of melanoma differentiation antigens via PKC and STAT3 activation, suggesting that Wnt5a may contribute to immune evasion (90). Wnt5A was

also found to enhance resistance to $BRAF^{V600E}$ inhibitor therapy (103). In summary, the phenotype switching model describes two populations of melanoma cells with anti-correlative melanocytic and mesenchymal gene expression patterns, which define the proliferative and invasive phenotype respectively, and phenotype switching is a potential resistance mechanism to targeted therapy and immunotherapy.

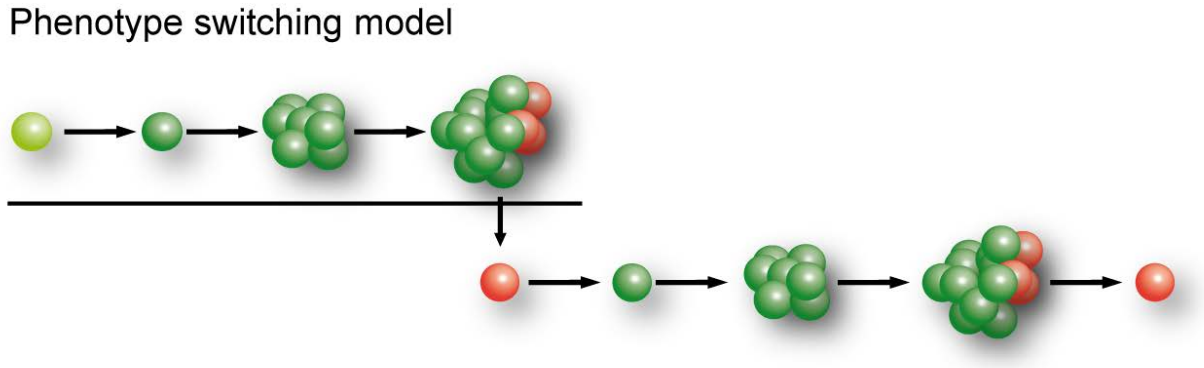

Figure 3. Melanoma phenotype switching model. The green spheres represent proliferative phenotype cells and the red spheres represent invasive phenotype cells. The model predicts that during tumor growth, most cells are in a proliferative state until some microenvironmental factors induce some cells to become invasive. These invasive cells leave the tumor bulk and establish a metastasis elsewhere by switching to a proliferative state.

Melanoma also possesses an innate trait to respond to extracellular cues provided by the extracellular environment which induces a phenotype switch in an embryonic environment (104). The ability for melanoma cells to adapt to such an environment highlights the high degree of plasticity melanoma cells are capable of. Other studies have also supported the plastic nature of melanoma (105, 106). Many groups have isolated and injected pure homogeneous populations of melanoma cells into immunocompromised mice and no matter which surface expression markers were selected, both positive and negative populations gave rise to tumors and the tumors were heterogeneous for the marker (107-110). As these pure populations of melanoma cells have the same genetic background, these results would suggest that the plasticity arises from reversible epigenetic mechanisms. Indeed, epigenetic mechanisms have been shown to play a role in melanoma heterogeneity and tumor growth. Roesch and colleagues have shown that a H3K4 demethylase, JARID1B, is required for continuous tumor growth and importantly JARID1B regulation is dynamic as JARID1B negative cells can become

positive and vice versa (109). Taken together, these studies provide compelling evidence for epigenetic involvement in the plasticity of melanoma to give rise to phenotypic heterogeneity.

DNA methylation and melanoma

DNA methylation provides a stable gene silencing mechanism involved in regulating gene expression and chromatin architecture. DNA methylation is defined by the addition of a methyl group to the cytosine of a cytosine-phosphate-guanine (CpG) dinucleotide. A family of DNA methyltransferases (DNMTs) catalyzes this reaction by transferring a methyl group from the donor S-adenosyl methionine (SAM) to cytosine at the fifth carbon position of the pyrimidine ring (111-113). The maintenance methyltransferase, DNMT1, preferentially catalyzes methylation of the unmethylated strand of hemimethylated DNA during DNA replication (111). The *de-novo* methyltransferases, DNMT3a and DNMT3b, establish new methylation patterns on both DNA strands during embryogenesis or differentiation processes (reviewed in (114)). CpG dinucleotides are not evenly distributed throughout the genome, but they are concentrated into regions called CpG islands. More than half of the genes (52%) of the human genome contain a CpG island in their promoter region (115). DNA hypermethylation of promoter CpG islands can permanently silence genes, and inactivation of tumor suppressor genes by promoter methylation is one event that can lead to tumor progression (116, 117), whereas DNA hypomethylation can reactivate latent retrotransposons, induce genome stability, and activate proto-oncogenes which can lead to tumor progression (118, 119). Thus aberrant DNA methylation has two potential oncogenesis mechanisms, hypermethylation and hypomethyation.

It is becoming increasingly apparent that DNA methylation plays an important role in melanoma development and progression (120). DNMT3a and DNMT3b are upregulated in metastatic melanomas compared to primary melanomas (121). Deng et al showed that DNMT3a is required for melanoma development and metastasis in a melanoma mouse model (122). Promoter hypermethylation has been detected for genes encoding factors involved in several signaling pathways critical to tumor progression, genes of notable tumor suppressor function include adenomatous polyposis coli (APC) (123), PTEN (124), O-6-methylguanine-DNA methyltransferase (MGMT) (125, 126), CDKN2A (127), RASS1FA (126, 128-130), and E-cadherin (CDH1) (126, 131). Genome-wide hypomethylation has also been observed in melanoma but little is known about its role in tumor initiation and progression (132). Specific areas of hypomethylaton have been detected at major repetitive DNA elements like LINE-1 and

Alu and at the melanoma antigen family A (MAGE-A) locus. However functional characterization of these genes has not been explored (133). Lian et al have shown that methylated cytosines are converted to hydroxymethylated cytosines by the ten eleven translocase (TET) family of dioxygenase enzymes in melanoma, and they functionally characterized this novel epigenetic marker and its impact on melanoma progression (134). Hydroxymethylated cytosines are the intermediate product of active demethylation of methylated cystosines to unmethylated cytosine and (135).

In summary, there is convincing evidence that DNA methylation plays an important role in melanoma by silencing tumor suppressors and increasing genomic instability by global hypomethylation.

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4. Aim of the thesis

Melanoma is the most aggressive skin cancer worldwide. Although melanoma is curable when detected in early stages of progression, patients diagnosed with late stage and metastatic melanomas have very poor survival. The difficulty in treating late stage and metastatic melanoma patients has been attributed to the heterogeneity of melanoma. However the molecular mechanisms of establishing heterogeneity are currently unknown. Our laboratory recently described the phenotype switching model for melanoma heterogeneity. This model hypothesizes that melanoma cells drive disease progression by switching between phenotypes of proliferation and invasion. Microarray experiments have shown that specific gene expression patterns differentiate between proliferative and invasive melanoma cell phenotypes. *In vitro* the proliferative and invasive phenotypes are morphologically distinct and differ in proliferation rate and invasiveness.

The aims of my PhD were:

1. Validate the phenotype switching model in other melanoma datasets
2. Investigate specific genes involved in phenotype switching
3. Investigate the role of DNA methylation in phenotype switching

In Chapter 5, we published a study describing our methodology in validating the two phenotype gene signatures in a large set of publically accessible microarray datasets on the Gene Expression Omnibus (GEO) array repository. This publication demonstrated that the gene expression signatures for the proliferative and invasive phenotypes established with our melanoma cell cultures were also seen in the majority of melanoma cell cultures used in other melanoma research laboratories worldwide.

In Chapter 6, we published a study describing the role of SOX9, a marker for the invasive phenotype, as a regulator for SOX10, a marker for the proliferative phenotype, in melanoma cells and in a *Tyr::Nras*^{Q61K} mouse model. SOX10 haploinsufficiency was previously shown to prevent naevi formation and hyperpigmentation in the *Tyr::Nras*^{Q61K} melanoma mouse model. In this publication, conditional deletion of SOX9 in both *Tyr::Nras*^{Q61K} *Sox10*$^{fl/-}$ and *Tyr::Nras*^{Q61K} *Sox10*$^{fl/fl}$ mice rescue the hyperpigmentation and naevi phenotype. In addition, SOX9 overexpression in melanoma cell cultures leads to down-regulation of SOX10 through the binding of SOX9 to the SOX10 promoter. This publication demonstrated the antagonistic roles

of SOX10 and SOX9 expression in melanoma development and potentially supports the phenotype switching model.

In Chapter 7, we published a study describing the landscape of DNA methylation between the proliferative and invasive phenotype, and functionally validated SOX9 as a marker for the invasive phenotype and correlate its expression and function with patient survival. SOX9 in chapter 6 was shown to suppress the proliferative phenotype by downregulation of SOX10. In this publication, DNA methylation regulates the expression of SOX9 and SOX9 is required for the invasive capacity of melanoma cells. Furthermore, high SOX9 expression was correlated with poor patient survival. This publication demonstrated the prognostic role of SOX9 in melanoma and the characterization of SOX9 in the invasive phenotype.

5. **Systematic classification of melanoma cells by phenotype-specific gene expression mapping**

Contribution

In this publication, I helped curate the datasets from the GEO database and perform some preliminary analysis on the proliferative and invasive gene signatures in the external datasets. I also performed the proliferation and invasion characterization experiments for a number of cell cultures. I was made second author for my contributions to this paper.

This study was published in *Pigment Cell Melanoma Research*; *Widmer et al. 2012.*

Summary

There is growing evidence that the metastatic spread of melanoma is driven not by a linear increase in tumorigenic aggressiveness, but rather by switching back-and-forth between two different phenotypes of metastatic potential. *In vitro* these phenotypes are respectively defined by the characteristics of strong proliferation/weak invasiveness and weak proliferation/strong invasiveness. Melanoma cell phenotype is tightly linked to gene expression. Taking advantage of this we have developed a gene expression-based tool for predicting phenotype called Heuristic Online Phenotype Prediction (HOPP). We demonstrate the predictive utility of this tool by comparing phenotype-specific signatures with measurements of characteristics of melanoma phenotype-specific biology in different melanoma cell lines and short-term cultures. We further show that 86% of 536 tested melanoma lines and short-term cultures are significantly associated with the phenotypes we describe. These findings reinforce the concept that a two-state system, as described by the phenotype switching model, underlies melanoma progression.

Significance

A recent model for melanoma progression suggests that melanoma cells switch back-and-forth between states of proliferation and invasion to drive disease progression. We describe the use of a new online expression-analysis tool which shows that melanoma cell expression signatures are divided into distinct groups correlating with behavioural phenotypes of proliferation or invasion. Using this tool we also show evidence suggesting that short term cultures, rather than cell lines, may be a more relevant model system for *in vitro* studies of melanoma.

Introduction

Cutaneous melanoma is an aggressively dangerous disease and median patient survival after metastases are diagnosed is only 6-9 months (Klimek et al., 2000). While recent

clinical trials have shown some improvements in overall survival for patients undergoing nonspecific immune or kinase inhibitor therapies, median survival rates remain dismal (Hodi et al., 2010, Flaherty et al., 2010, Chapman et al., 2011). Therefore gaining a comprehensive cellular and molecular understanding of metastatic spread continues to be critically important. A major focus of study is the phenomenon of melanoma cell heterogeneity. That there can be significant molecular differences between melanoma cells from the same lesion is widely appreciated, though what this means for disease progression remains the subject of debate (Shackleton and Quintana, 2010, Hoek and Goding, 2010, Roesch et al., 2010).

Pursuing *in vitro* studies into melanoma heterogeneity, we and others have identified two subgroups of cultured melanoma cells which are clearly distinguishable by how they express specific genes (Bittner et al., 2000, Hoek et al., 2006, Jeffs et al., 2009, Alexaki et al., 2010, Javelaud et al., 2011). *In vitro* experiments have identified one subgroup as a *proliferative* phenotype and the other as an *invasive* phenotype, and immunohistochemical testing has shown that individual primary and metastatic lesions typically include cells of both phenotypes (Eichhoff et al., 2010, Eichhoff et al., 2011). Their co-presence in lesions is likely explained by experiments which have shown that melanoma cells can switch between phenotypes *in vivo* (Hoek et al., 2008a). Together these findings prompted what has become the phenotype switching model for melanoma progression, in which melanoma cells respond to changing microenvironmental signals by reprogramming their gene expression to switch between states of proliferation and invasion (Hoek et al., 2008a, Pinner et al., 2009, Hoek and Goding, 2010). In addition to providing a mechanism for the metastatic cascade and explaining heterogeneity within a lesion, phenotype switching may also underlie targeted therapy escape. For example, slow-cycling invasive phenotype melanoma cells would be less susceptible to standard chemotherapies and such survivors could later switch to a proliferative state (Fukunaga-Kalabis et al., 2011). Furthermore, cultures of invasive and proliferative phenotype cells have been shown to differentially express melanocytic markers, suggesting that subverting the immune response to specifically target melanocytic cells may be similarly defeated (Hoek et al., 2006). Finally, we have shown that while proliferative phenotype cells respond to MAPK inhibitors, invasive phenotype cells do not and may provide a pool from which resistant cells emerge (Zipser et al., 2011).

Most recently, our group showed that microenvironmentally-induced changes in the expression patterns of specific LEF/TCF family transcription factors may be important for the phenotype switching mechanism (Eichhoff et al., 2011). That the microenvironment is involved in phenotype switching is corroborated by the results of other groups who have explored its influence on melanoma gene expression and metastatic potential (Seftor et al., 2006, Folberg et al., 2006, Postovit et al., 2008). Several laboratories have considered the phenotype switching model and reported data supporting a relationship between factors expressed in a phenotype-specific manner and disease progression (Carreira et al., 2006, Almanzar et al., 2009, Orgaz et al., 2009, O'Connell et al., 2009, Alexaki et al.,2010). These studies contrast those which purport to identify melanoma stem cells (or melanoma initiating cells) by the identification of stem-cell markers (Fang et al., 2005, Schatton et al., 2008). While

the melanoma stem cell paradigm also seeks to explain intralesional heterogeneity and therapy escape, several lines of research indicate that stem cell markers are not an exclusive or even necessary prerogative of cells propagating metastatic disease (Quintana et al., 2008, Roesch et al., 2010, Cheli et al., 2011). The inference is that "stemness" is a reversible phenotype to be adopted or shed by melanoma cells in response to microenvironmental signalling (Hoek and Goding, 2010). The reversible nature of stem cell marker expression closely mirrors the concept of phenotype switching, and we have argued that invasive phenotype melanoma cells themselves fit multiple criteria for cancer stem cells (Hoek and Goding, 2010). Thus it is critical that researchers working with melanoma cells are both aware of and can account for the phenotypes as they study various aspects of melanoma biology.

We have designed a gene expression analysis algorithm, termed Heuristic Online Phenotype Prediction (HOPP), which uses archetypes of proliferative and invasive phenotype signatures to identify and predict the phenotypes of melanoma cell lines and cultures. We tested its utility by carrying out proliferation and invasion experiments on both short-term melanoma cultures and widely-used melanoma cell lines. We also used HOPP to carry out a phenotyping survey of several hundred published expression profiling experiments which showed that, contrary to the expectation that samples may be uniformly distributed throughout the "expression space" between phenotypes, more than 80% of samples cluster with either the proliferative or the invasive phenotype.

Results

Melanoma phenotype specific gene expression

For high throughput studies of melanoma gene expression, the most frequently employed platform remains the HG-U133 series produced by Affymetrix (Hoek, 2007). In our experience this platform has proven to be a robust tool for genome-wide expression studies (Hoek et al., 2006, Zipser et al., 2011). Therefore the current study sources datasets produced using HG-U133 series platforms. We used six different datasets (including a total of 218 melanoma lines and cultures) to derive the phenotype signature standards. For each dataset we performed a class-discovery analysis following methods previously described (Hoek et al., 2006), with the modification that we used bootstrapped confidence estimates for determining phenotype membership based on sample clustering (Kerr and Churchill, 2001). From this we identified 100 samples in one subgroup and 92 samples in a second subgroup (Appendix; Table S1). A further 26 samples did not show 100% stability in their cluster memberships and were therefore not used to identify genes with subgroup-specific expression patterns. With the subgroup assignments we used ANOVA to identify a set of 97 genes expressed differentially between the two subgroups (Appendix; Table S2). Reference to earlier works with similar expression signatures derived from smaller datasets indicated that the first subgroup are characterized by a proliferative phenotype signature and the other by an invasive phenotype signature (Hoek et

al., 2006, Jeffs et al., 2009). The identified list of genes is thus referred to as the Melanoma Phenotype-Specific Expression (MPSE) list.

Generating MPSE correlation data

Normalization of high throughput data across samples within a dataset is principally designed to account for dataset-specific sources of technical variation. However, different datasets do not necessarily share equivalent characteristics of variation and it is impractical to expect that analyses of combined datasets would not be susceptible to these differences. Our aim was to devise a method for predicting phenotype from gene expression data for single-sample experiments (where cross-sample normalization procedures do not apply) as well as for multi-sample experiments (considering each sample independently). We extracted the raw signal intensities for 134 probe-sets corresponding to the 97 MPSE genes from 192 samples (the control set) representing 100 proliferative and 92 invasive phenotype signatures (Table S1). For each phenotype we then calculated the median cross-sample values for every probe-set. This yielded two phenotype-specific standard signatures (Table S2). An online algorithm, Heuristic Online Phenotype Prediction (HOPP, http://www.jurmo.ch/hopp), was written to perform Pearson's correlation analyses comparing data from each phenotype- assigned sample against both the invasive and the proliferative standards, yielding two correlation coefficients per sample. We graph the function of the two correlation coefficients on a simple cartesian coordinate system where the x-axis describes correlation with the proliferative phenotype signature standard, and the y-axis describes correlation with the invasive phenotype signature standard, referring to this particular arrangement as a "Widmer plot".

Plotting of sample correlation coefficients showed group-specific clustering of proliferative and the invasive phenotype samples (Figure 1A; Table S3). We used this data to calculate the probability density distribution, which also showed group-specific clustering of the samples and indicated a strong peak near the proliferative standard (Figure 1B). We acquired DNA microarray data for an additional 318 *in vitro* melanoma gene expression profiling experiments from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) and used HOPP to also calculate and plot their sample correlation coefficients. This revealed a distribution closely similar to that of the control set (Figure 1C; Table S4). Calculating the probability density distribution reveals similar results as obtained with the control set (Figure 1D). Including the highly stringent Bonferroni multiple testing correction adjustment, we calculate the significance of a Pearson's correlation (r) of 0.4 to be $P < 0.001$. This shows that 81% of samples have expression signatures which are closely correlated ($r > 0.4$) to only one standard. In comparison, 1% of samples were closely correlated to both and 18% were not correlated to either. It should be appreciated that, because of the high number of samples being examined (the panels in Figure 1 describe the results obtained from a total of 536 profiling experiments) there is a high likelihood that some of these apparent outliers are the result of technical fault. For example, common faults that may be expected to affect the outcome on a Widmer plot include the use of degraded RNA samples to perform gene

profiling experiments, or using samples which are contaminated with additional cells from other sources.

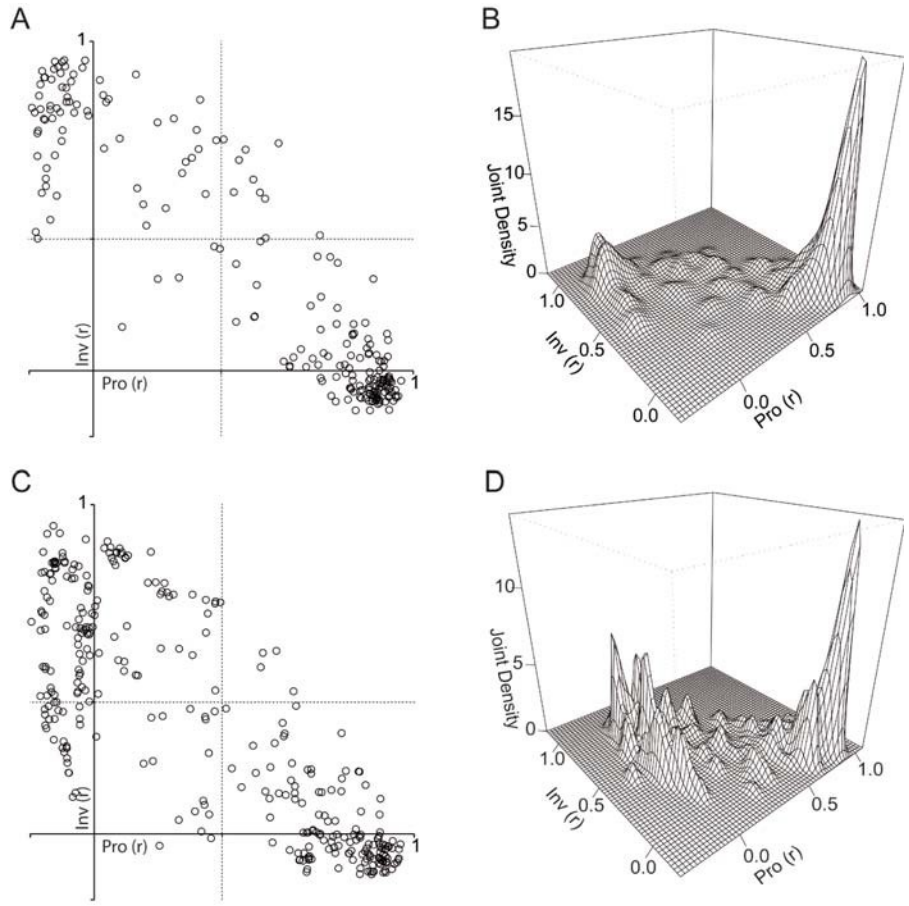

FIGURE 1. *MPSE correlation plots.* Sample gene expression data for the MPSE gene set was used to calculate, via HOPP, correlation coefficients (r values) against the proliferative and invasive standard signatures. (A) Widmer plot (proliferative (x-axis) *versus* invasive (y-axis) correlation coefficients) of the control data. This shows that 93.2% of samples yielded r > 0.4 for one phenotype only, 4.5% yielded r > 0.4 for both phenotypes and 2.3% yielded r > 0.4 for neither. (B) The kernel density estimation of the probability distribution for control data shows distinct sample concentrations near each phenotype standard. (C) Widmer plot of 318 test samples. This shows that 81.4% of samples yielded r > 0.4 for one phenotype only, 1.2% yielded r > 0.4 for both phenotypes and 17.4% yielded r > 0.4 for neither. (D) The kernel density estimation of the probability distribution for the test samples also shows distinct sample concentrations near the phenotype standards.

Assessing phenotype prediction for melanoma cultures and cell lines

We performed DNA microarray gene expression profiling on twelve additional short-term melanoma cell cultures and used HOPP to predict their phenotypes (Figure 2A). We then

tested their *in vitro* characteristics of proliferation and invasion. Under standard culturing conditions the six proliferative signature samples had an average population doubling time of 42.9 h, while the six invasive signature samples had an average population doubling time of 131.3 h (Figure 2C). In invasion assays the proliferative signature samples showed an average invasive index of 3.2%, while the invasive signature samples had an average invasive index of 30.3% (Figure 2E). These differences in proliferation and invasion were significant ($P < 0.002$ and $P < 0.004$, respectively). Culturing of these cells on Matrigel, as described previously (Zipser et al., 2011), yields surface organization patterns which are phenotype specific. Proliferative phenotype melanoma cells adopt an organization of small and isolated clusters, while invasive phenotype cells form connected networks (Appendix; Figure S1). Culturing short-term melanoma cell cultures on Matrigel confirmed phenotype-specific surface organization patterns (Table 1).

Table 1. Combined summary data for melanoma cell cultures.

Cell cultures	Accession	Correlation Pro (r)	Correlation Inv (r)	Doubling time (h)	Invasion (%)	Matrigel[a]
M080423	GSM700746	0.831	-0.122	70.9	5.3	isolated
M000921	GSM700745	0.550	0.050	42.4	7.8	isolated
M010817	GSM700743	0.734	-0.046	42.9	0.9	isolated
M980513	GSM700742	0.891	-0.074	37.1	0.0	isolated
M050829	GSM700744	0.618	0.215	33.3	3.0	isolated
M000907	GSM108375	0.511	0.260	30.5	2.4	isolated
M060125	GSM700750	-0.121	0.806	184.0	30.4	connected
M061103	GSM700749	-0.131	0.769	164.9	42.1	connected
M081008	GSM700752	-0.147	0.718	73.6	11.8	connected
M080310	GSM833481	-0.154	0.773	159.8	13.6	connected
M080201	GSM833482	-0.187	0.685	145.9	20.8	connected
M080214	GSM833483	-0.178	0.774	59.3	63.4	connected

[a] see Figure S1 for explanatory examples.

Table 2. Combined summary data for melanoma cell lines

Cell line	Accession	Correlation Pro (r)	Correlation Inv (r)	Doubling time (h)	Invasion (%)	Matrigel[a]
888mel	GSM206439	0.802	-0.052	27.8	8.8	isolated
WM983A	GSM109047	0.878	-0.027	46.7	2.8	isolated
WM983B	GSM109048	0.860	-0.052	49.0	0.9	isolated
501mel	GSM555120	0.831	-0.012	22.8	7.8	connected
SK-MEL-28	GSM206543	0.947	-0.082	24.5	1.6	connected
WM793B	GSM109043	-0.119	0.939	39.0	20.0	connected
WM852	GSM109044	-0.134	0.804	41.0	15.9	connected
1205Lu	GSM109021	-0.088	0.924	24.2	5.4	connected

[a] see Figure S1 for explanatory examples.

We compared the short-term culture results with a similar analysis of eight widely-used melanoma lines (SK-MEL-28, 501mel, 888mel, WM793, WM852, WM983A, WM983B and 1205Lu). Each of these has already been subject to microarray expression profiling by other researchers and this data is available on the NCBI GEO database. However, we performed additional DNA microarray expression profiling experiments on these lines and used HOPP to predict their phenotypes (Figure 2B). This showed close agreement between the published data and our experiments, where five are proliferative signature lines (888mel, WM983A, WM983B, 501mel, SK-MEL-28) and three are invasive signature lines (WM793B, WM852, 1205Lu). Then we assessed their *in vitro* proliferative and invasive characteristics. We found for these cell lines that while there was no significant difference in population doubling times between proliferative (34.1 h) and invasive (34.7 h) signature lines (Figure 2D), there was a significant difference ($P < 0.026$) in invasiveness, with proliferative signature lines showing an invasive index of 4.4% and invasive signature lines showing an invasive index of 13.8% (Figure 2F) in line with previous observations (Alexaki et al., 2010). However, culturing of cell lines on Matrigel yielded inconsistent results, with two (501mel, SK-MEL-28) yielding outcomes which were contra to HOPP prediction (Table 2, Figure S1).

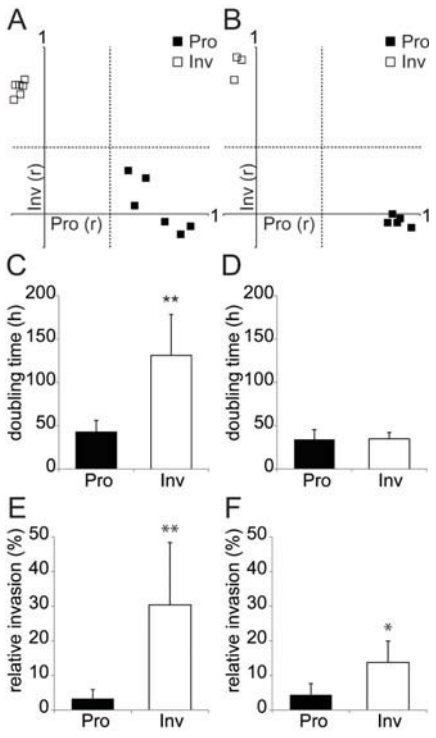

FIGURE 2. Phenotype prediction of *in vitro* behaviours. (A) Widmer plot of HOPP data from short- term cultures of melanoma cells. (B) Widmer plot of HOPP data from melanoma cell lines. (C) In vitro doubling time (h) of short-term cultures of melanoma cells. (D) In vitro doubling time (h) of melanoma cell lines. (E)

In vitro invasive indices of short-term cultures of melanoma cells. (F) In vitro invasive indices of melanoma cell lines (*P < 0.05, **P <0.01). Dotted lines in the Widmer plots indicate r = 0.4.

Phenotype is independent of BRAF mutation status

The $BRAF^{V600E}$ mutation is a frequent occurrence in melanoma, being present in more than half of all samples tested (Davies et al., 2002). The involvement of this mutation in disease progression has shown BRAF to be a promising target molecule in therapeutic trials (Flaherty et al., 2010, Bollag et al., 2010). We therefore explored whether the BRAF mutation had any relationship with the proliferative and invasive phenotype signatures. Four different datasets (GSE10916, GSE4841, GSE4843 and GSE7127) supply genotyping information concerning the mutation status of BRAF. HOPP analyses of these show there is no significant association between the HOPP result and BRAF mutation status (Figure 3). This indicates that BRAF mutation status has no relationship with phenotype-specific gene expression.

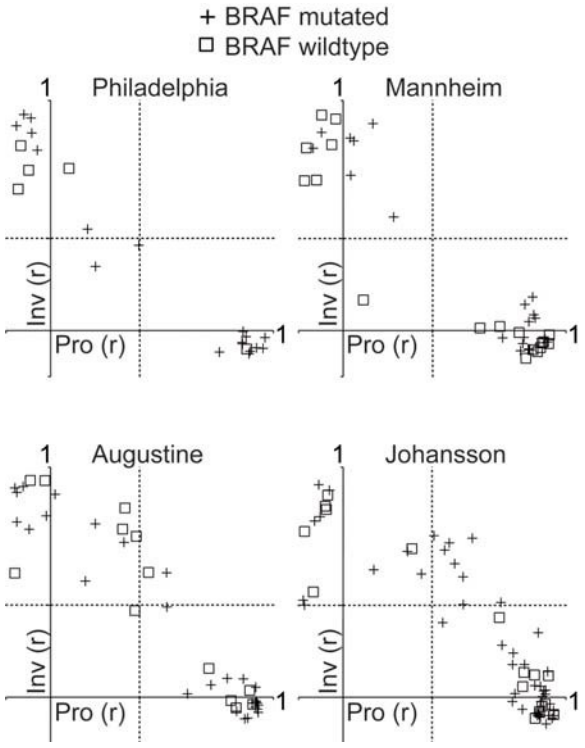

FIGURE 3. Phenotype and BRAF mutation. Datasets for which BRAF mutation status data was available were subjected to HOPP analysis and the distributions of BRAF wild-type versus BRAFV600E were assessed. Widmer plots of four different datasets reveals no significant relationship between BRAF mutation status and HOPP outcome. Dotted lines indicate r = 0.4.

Melanoma cell phenotypes in vivo

In vitro, melanoma cell phenotype is assumed to be relatively homogenous and it is supposed that nearly all cells in a given culture share a uniform level of gene expression. In contrast to this, *in vivo* observation of melanoma tumors reveal a heterogeneous distribution of many markers as tumors typically include an irregularly distributed composition of tumor, stromal and infiltrating immune cells. The distribution of marker genes specific to invasive or proliferative phenotypes suggest that tumors, and the biopsies taken from them, also include cells of both phenotypes in proportions which are difficult to predict. Subsequently, while the measurement of the expression of any given gene is its mean among all cells in a biopsy, it does not necessarily follow that this is representative of the entire lesion. Thus tumor heterogeneity, with its irregular distribution of cell phenotypes, likely complicates the drawing of clinically relevant conclusions from expression data obtained from either biopsies or derived cell cultures. We therefore examined data obtained from melanoma tissue samples and used HOPP to perform Pearson correlation analyses against the proliferative and invasive standards. For example, Riker and co-workers published a study in which, among other things, they compared primary melanomas against metastatic lesions (Riker et al., 2008). We found that HOPP analysis does not separate these sample classes, with both primaries and metastases being similarly spread between proliferative and invasive phenotype signatures (Figure 4A). This supports earlier data which indicated that primary and metastatic lesions are composed of irregular distributions of both phenotypes (Hoek et al.,2006, Eichhoff et al., 2011).

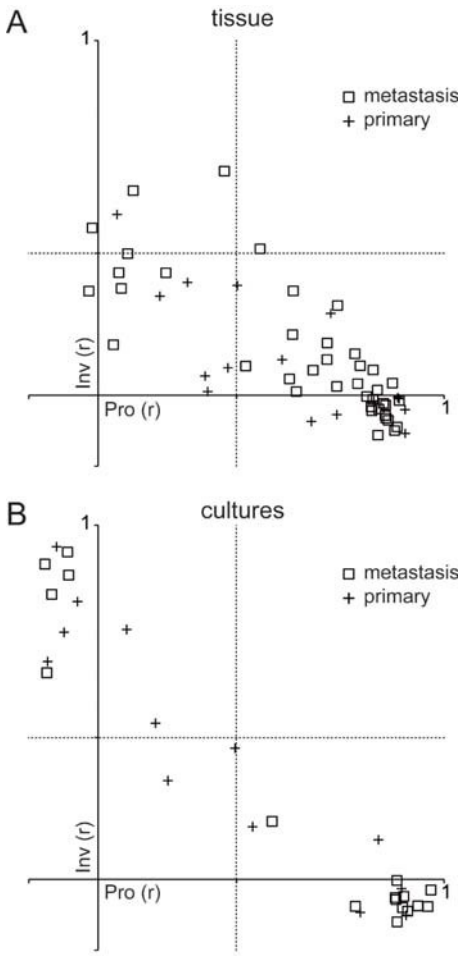

FIGURE 4. Primary versus metastatic melanoma tissues and cultures. (A) The Riker dataset (GSE7553) was assessed with HOPP and is shown here as a Widmer plot with samples identified according to whether they are primary melanoma tissue (crosses) or metastatic melanoma tissue (squares). (B) The Philadelphia dataset (GSE4841) was assessed with HOPP and the results are displayed here as a Widmer plot with samples identified according to whether they are primary melanoma cells (crosses) or metastatic melanoma cells (squares). Neither primary nor metastatic melanoma samples reveal a significant association with the phenotype signatures. Dotted lines indicate r = 0.4.

Comparison with hierarchical clustering

Freedman and co-workers profiled gene expression in cultures of primary and metastatic melanoma lesions and categorized them according to unsupervised hierarchical clustering (Freedman et al., 2011). Re-analysis of this data using HOPP shows that their M1 and M3 subclasses fit discretely into the proliferative and invasive phenotype signatures, respectively (Figure 5).

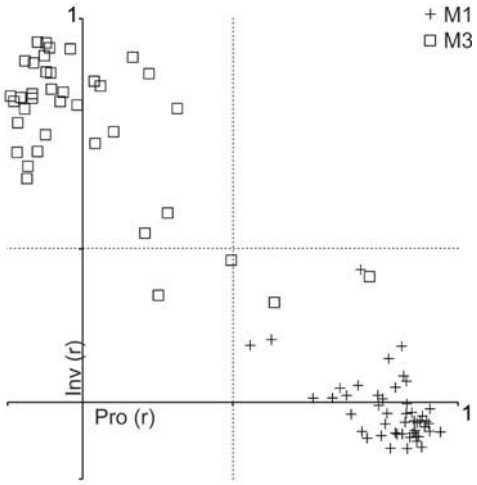

FIGURE 5. Hierarchical clustering versus HOPP. The Freedman data was assessed with HOPP and the results are displayed here as a Widmer plot with cultured samples identified according to the M1 (crosses) and M3 (squares) clustering results obtained by Freedman and co-workers. Dotted lines indicate r = 0.4.

Interlaboratory phenotype signature consistency

We compared HOPP analyses for nine different cell lines where for which were at least three independent sources of expression profiling data. Widmer plots of the results showed that four cell lines (WM35, MDA435, A2058 and A375) demonstrated inconsistent phenotype signatures. In contrast, five cell lines (SK-MEL-28, C8161, MALME-3M, SK-MEL-19 and SK-MEL-5) showed consistent phenotype signatures (Figure 6).

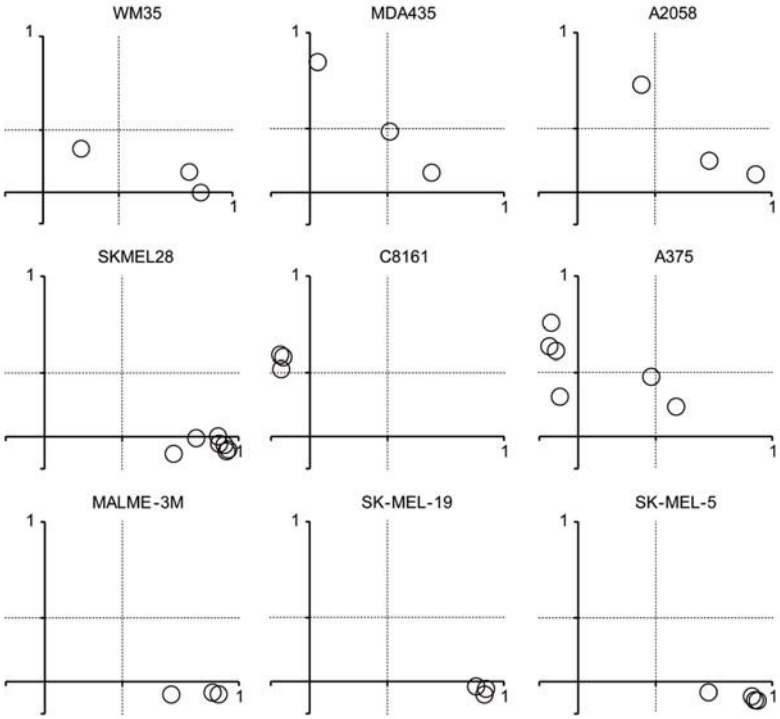

FIGURE 6. Melanoma cell line expression signature inconsistencies. The results of HOPP analyses of data taken from four different cell lines in wide use, each of which was expression profiled by at least three different laboratories, are shown here as Widmer plots. These data show that melanoma lines WM35, MDA435 and A2058 show little inter-laboratory consistency in their gene signatures. On the other hand, seven different laboratories yielded SK-Mel-28 expression data which showed a strongly consistent proliferative phenotype signature. Dotted lines indicate r=0.4.

Discussion

Previous studies which identified two phenotypes of melanoma cell yielded some clues concerning the molecular nature of phenotype switching (Hoek et al., 2006, Carreira et al.,2006). However, it was not clear if there were distinct intermediate stages between the proliferative and invasive archetypes. We show here that a large majority of gene expression profiles derived from melanoma *in vitro* cluster very closely to one or the other phenotype standard. This argues against the idea that melanoma cells adopt clear intermediate states during phenotype switching and counters an earlier suggestion that melanoma cells may inhabit a “continuum” in the expression space between phenotype standards (Hoek et al., 2008a).

We tested the ability of HOPP to predict *in vitro* behaviours in a dozen short-term cultures of melanoma cells and eight widely-used melanoma cell lines (Figure 2). We found close correlation between HOPP clustering and the *in vitro* proliferative and invasive characteristics of short-term cultures. This showed a close association between specific gene expression patterns and *in vitro* biological activities of short-term cultures of melanoma cells, meaning that HOPP may be used with confidence to predict their

characteristics. Similarly, our *in vitro* characterization of cell lines showed that *in vitro* invasiveness correlated closely with phenotype specific expression patterns. However, we found no such significant signature-specific difference between cell lines' rates of proliferation. Overall, cell lines of both phenotypes proliferated significantly faster than invasive phenotype cell cultures, and were at least as fast as proliferative phenotype cell cultures. Why cell lines proliferate so well may be explained by the effects of somatic changes and experimenter selection. Cells in culture likely remain subject to additional somatic changes which could contribute towards the selection of faster growing clones. For example, such changes may influence proliferation-critical processes such as cyclin degradation, where it has been shown that truncation of cyclin genes abrogates normal processes nuclear expulsion and cytosolic degradation (Van Dross et al., 2006). Furthermore, cells which did not proliferate well in culture were routinely discarded on the basis that they were probably not cancer cells. This is understandable with a clonal evolution model in which cancer cells are thought to increase characteristics of both proliferation and invasion as the disease progresses (Miller and Mihm, 2006). The differences between short-term cultures and cell lines, in which the phenotypic behaviours of cell lines are less correlated with their expression signatures, suggest that over the long period of time cell lines have been in circulation they may have deviated from their original characteristics and been selected for by researchers' expectations of what constituted good *in vitro* models.

Many melanoma researchers use cell lines or cultures to study the disease. As mentioned earlier, a fundamentally important assumption had been that melanoma cells may be differentiated by their potential to drive metastatic progression. The language used to convey the extent or degree of this potential in a given cell line or culture is varied. Descriptors are sometimes determined by the clinical stage of the lesions from which cells are derived. This can influence conclusions drawn on the basis that differences between lines are significantly correlated with interpretations in which clinical stage reflects aggressiveness. For example, Pochec and co-workers use the fact that A375 comes from a metastasis and WM35 comes from a primary to make the claim that differences in how these cells glycosylate an integrin is significant for the acquisition of invasiveness (Pochec et al., 2003). Similarly, the Wistar Institute Melanoma Research Center maintains a collection of melanoma cell lines which are explicitly characterized according to clinical stages of the patient lesions from which they are derived (http://www.wistar.org/lab/meenhard-herlyn-dvm-dsc/page/resources). However, our previously published analyses (Hoek et al., 2006) found no gene expression pattern correlating with stage progression in these lines and HOPP analysis confirms that their distribution is not phenotype specific. Alternatively, descriptors may be derived experimentally, for example cells may be described as "metastatic" or "non-metastatic" according to their performance in animal models (de Wit et al., 2005). In either context, differences between "metastatic" and "non-metastatic" are frequently interpreted in the same way as being intrinsic to disease progression. However, this is problematic because a cell line derived from a metastasis may not necessarily produce metastases in an animal model.

A significant consequence of a paradigm in which the existence of two distinct phenotypes of melanoma cell goes unrecognized is the likelihood of large-scale study bias towards one

or another phenotype. For example, scientists studying DNA methylation patterns in melanoma examined eleven different cell cultures (Bonazzi et al., 2011). Of these, we note (from Table S1) that nine have clear proliferative phenotype signatures and one has a clear invasive phenotype signature. It is probable that the reason for this is simply because proliferative phenotype signature melanoma cultures grow faster and are thus more amenable to *in vitro* research than slower-growing invasive phenotype cultures. The results of these researchers' experiments are therefore biased toward proliferative phenotype cells. Considering our probability density calculations it is clear that a large majority of experiments have been conducted on proliferative phenotype cultures, suggesting that this bias is wide-spread.

Interestingly, other workers have performed expression profiling analyses and clustering experiments which yielded distributions of data strikingly similar to the partition described by phenotype switching. For example, Freedman and co-workers profiled gene expression in cultures of primary and metastatic melanoma lesions and categorized them according to unsupervised hierarchical clustering (Freedman et al., 2011). Our analysis of this data showed that their M1 and M3 subclasses fit into the proliferative and invasive phenotype signatures, respectively. By gene enrichment analyses and pathway activity analyses these researchers identified distinct patterns of pathway activation. The M1 subclass exhibited stronger activity of the β-catenin, EGFR and ER pathways while the M3 subclass showed higher activity of SRC and STAT3 pathways. STAT3 signalling is known to induce expression of MMP2 and VEGF, genes which are highly upregulated in the invasive phenotype (Niu et al., 2002, Wei et al., 2003, Xie et al., 2004). Interestingly, due to the activation of STAT3 in the M3 subclass and the reported importance of STAT3 activation for the formation of metastases in a mouse model (Xie et al., 2004), Freedman and co-workers suggested that the M3 subclass is a more aggressive subset. This is important because it relates to how the meaning of a term like "aggressive" is contextually nuanced. For example, it is implied that the M1 subset, which shows higher EGFR pathway activity, is less aggressive. This interpretation contrasts with other studies which report that EGFR pathway activation is critical for promoting tumor growth and metastasis, and which conclude that EGFR pathway activation is characteristic of aggressive cells (Ueno et al., 2008, Schartl et al., 2010). Our own interpretation is that these results are not in conflict because they are likely dealing with different phenotypic states. In this context, STAT3 signalling is important for driving invasiveness, while EGFR signalling is critical for proliferation. As both phenotypes are required for metastatic spread it is understandable that suppression of either pathway would retard the aspects of disease progression to which they respectively apply.

In a previous study we examined the effects of MAP kinase inhibition on proliferative and invasive phenotype melanoma cells (Zipser et al., 2011). While this showed that response to MAP kinase inhibition was phenotype-specific, we also saw evidence for the involvement of MAP kinase activity in phenotype regulation. Specifically, we noted that MAP kinase inhibition of proliferative phenotype cells induced the acquisition of invasive phenotype characteristics, which would revert upon removal of the inhibitor. Though expression profiling was performed on these samples, we found that MAP kinase

inhibition induced no significant change in gene expression and analysis by HOPP confirmed this (data not shown). The transience of the induced changes seen suggests how melanoma cells *in vitro* maintain their phenotype. Under standard culturing conditions neither proliferative phenotype cells nor invasive phenotype cells have been observed to undergo phenotype change (Hoek et al., 2006). This may seem inconsistent with a phenotype switching model which holds that microenvironmental influence is key to phenotype change (Hoek et al., 2008a). However, we suggest that the particular microenvironmental influences required for phenotype change are absent under standard culturing conditions, allowing different phenotypes to persist (Eichhoff et al., 2011).

Scientists have been culturing cancer cells (including melanoma) from patient materials for a century (Losee and Ebeling, 1914) and immortalized cancer cell lines have been available for sixty years (Gey et al., 1952). Accordingly, there are many human melanoma cell lines which have been in common use for decades. For example, SK-Mel-28 and A375 are widely recognized examples of "standard" melanoma lines that have been in circulation since the 1970s. While it is a general assumption that any given aliquot is representative of a cell line, passage through the years (and many hands) can lead to confusion. Accordingly, we have found that distinct batches of a cell line can yield inconsistent results when examined using the HOPP algorithm. It is important that these results show that many cell lines are consistent in their phenotype signature, and while this doesn't prove the identity of a cell line, it is circumstantial evidence to that effect and at the very least indicates that it the phenotype it is supposed to be. For cell lines where there is little agreement in HOPP results (e.g. A375) one is left to wonder which are the exemplars and which have become compromised.

Finally, the principles of HOPP can be readily applied to any system where a pair of standard signatures may be used in the analysis of single samples. So long as the standards were generated with sufficient samples to establish signatures with statistical confidence, then subsequent samples may be measured against such standards in the same way as we have described here for melanoma cells. While the MPSE standards for proliferative and invasive melanoma cell phenotypes are the default for HOPP, users may upload their own standards instead.

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Supporting Information

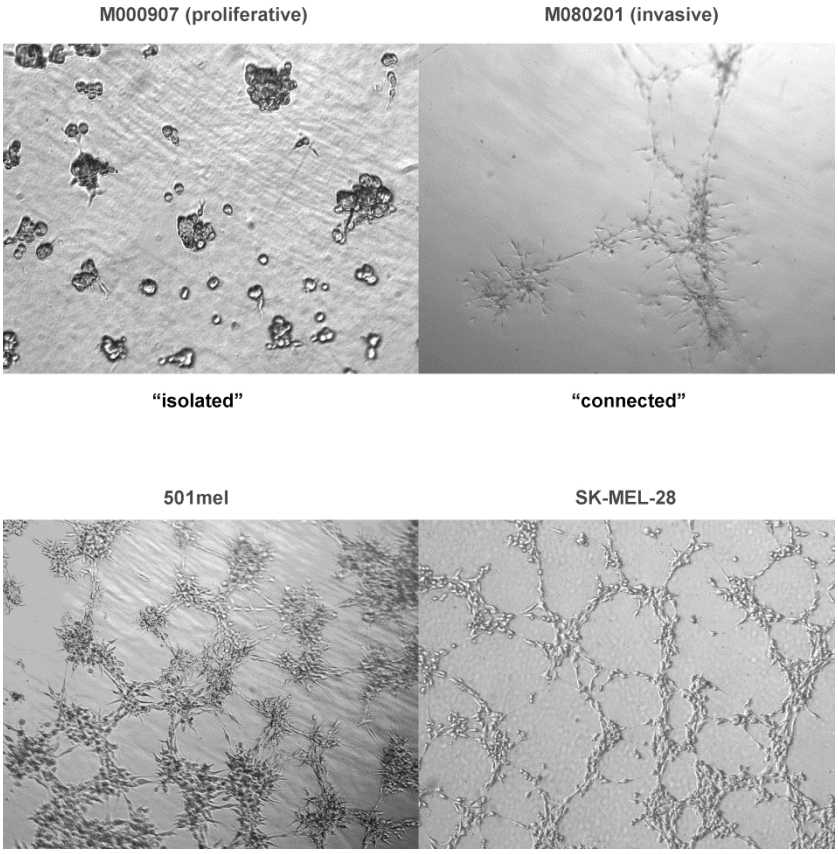

Figure S1. Phenotype-specific Matrigel surface organization patterns

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6. Antagonistic cross-regulation between Sox9 and Sox10 controls an anti-tumorigenic program in melanoma

Contribution

In this publication, I performed all the *in vitro* experiments involving characterization of SOX10 and SOX9 in melanocytes, naevi cells, and melanoma cells, knockdown of SOX10 and SOX9 in melanoma cells, and the generation of SOX9 overexpressing melanoma cells. I also analyzed the gene expression microarray data for the SOX9 overexpressing melanoma cells. I was made second author for my contributions to this paper.

This study was published in *PLoS Genetics; Shakhova et al. 2015.*

Abstract

Melanoma is the most fatal skin cancer, but the etiology of this devastating disease is still poorly understood. Recently, the transcription factor Sox10 has been shown to promote both melanoma initiation and progression. Reducing SOX10 expression levels in human melanoma cells and in a genetic melanoma mouse model, efficiently abolishes tumorigenesis by inducing cell cycle exit and apoptosis. Here, we show that this anti-tumorigenic effect functionally involves SOX9, a factor related to SOX10 and upregulated in melanoma cells upon loss of SOX10. Unlike SOX10, SOX9 is not required for normal melanocyte stem cell function, the formation of hyperplastic lesions, and melanoma initiation. To the contrary, SOX9 overexpression results in cell cycle arrest, apoptosis, and a gene expression profile shared by melanoma cells with reduced SOX10 expression. Moreover, SOX9 binds to the SOX10 promoter and induces downregulation of SOX10 expression, revealing a feedback loop reinforcing the SOX10 low/SOX9 high anti-tumorigenic program. Finally, SOX9 is required *in vitro* and *in vivo* for the anti-tumorigenic effect achieved by reducing SOX10 expression. Thus, SOX10 and SOX9 are functionally antagonistic regulators of melanoma development.

Author Summary

For the development of future cancer therapies it is imperative to understand the molecular processes underlying tumor initiation and expansion. Many key factors involved in these processes have been identified based on cell culture and transplantation experiments, but their relevance for tumor formation and disease progression in the living organism is often unclear. Therefore, genetically modified mice spontaneously developing tumors present indispensable models for cancer research. Here, we address this issue by studying the formation of melanoma, the most fatal skin tumor in industrialized countries. To this end, we use a transgenic mouse model to elucidate cellular and molecular mechanisms regulating congenital nevus and melanoma initiation. We show that a transcription factor called SOX10 promotes melanoma formation by repressing an anti-tumorigenic program involving the activity of a related factor, SOX9. When SOX10 is inactivated, SOX9 becomes upregulated and induces cell cycle arrest and death in melanoma cells. Furthermore, upon experimental elevation of SOX9 levels, SOX10 activity is suppressed, revealing an antagonistic relationship

between SOX9 and SOX10 in melanoma initiation. Knowledge of how an anti-tumorigenic program can be stimulated by modulating the activities of these key factors might help to design novel therapeutic strategies.

Introduction

Sox (Sry (sex determining region Y)-related HMG box) genes encode a family of transcription factors that are characterized by a conserved high-mobility group (HMG) domain mediating their binding to DNA in a sequence-specific manner [1-3]. While the majority of Sox proteins functions as transcriptional activators, some members of the Sox family including Sox9 and Sox10 may also act as transcriptional repressors [4-6]. *Sox* genes play key roles in embryonic development and are major determinants of stem cell behavior, regulating cell fate decisions and maintaining cellular identity [3]. Their crucial role in normal tissue formation and homeostasis is evident from the fact that several mutations in Sox genes are causative for developmental diseases, and accumulating evidence demonstrates the important functional role of Sox family proteins in a variety of cancers [7-10].

A common feature of SoxE group proteins, which includes Sox9 and Sox10, is their expression in neural crest (NC) cells during embryonic development [2,11]. NC cells are a transient embryonic cell population that gives rise to most of the peripheral nervous system, chondrocytes and osteoblasts of craniofacial structures, smooth muscle cells of the cardiovascular system, and melanocytes, the pigmented cells of the skin [12]. While Sox9 is expressed in premigratory NC cells and in the pharyngeal apparatus, Sox10 is found in NC cells at the time of their emigration and is essential for their self-renewal and survival [12-16]. Loss of *Sox10* results in absence of most NC derivatives, whereas *Sox10* haploinsufficiency causes Waardenburg Hirschsprung syndrome, characterized by aganglionic megacolon, pigmentary abnormalities and often deafness due to loss of sensory innervation [13, 17-20]. In the melanocytic lineage, Sox10 is expressed during all stages of development as well as in the adult and is required in different species for the generation and homeostasis of embryonic and adult melanocytes *in vitro* and *in vivo* [13, 21-25].

In contrast, loss of Sox9 in the NC does not lead to general defects in NC-derived structures, but specifically affects the development of mesectodermal derivatives, such as smooth muscle cells and craniofacial bones and cartilage [11,26-28]. Furthermore, heterozygous mutations in *Sox9* in both mice and humans, result in campomelic dysplasia, a syndrome associated with dwarfism, skeletal malformations, cleft palate, XY sex reversal and often hermaphroditism [28- 30]. However, data on Sox9 expression in melanocytes are inconsistent, and a functional implication of Sox9 in melanocyte formation has not been provided so far [23].

Based on the assumption that mechanisms of tumor formation might be related to those underlying the generation of the cell type, from which the tumor develops, we and others have recently addressed the function of Sox10 in melanoma. These studies demonstrated a crucial role of Sox10 in the pathogenesis of giant congenital naevi and melanoma in both mice and humans by regulating proliferation and survival of melanocytic cells and maintenance of their

cellular identity [9; 31]. However, the precise molecular mechanisms mediating Sox10 function in melanoma remain to be investigated.

Here we show that in contrast to *Sox10*, *Sox9* appears to be expressed at very low levels only and is functionally not required in melanocyte stem cells, committed melanocytes, and melanoma cells. However, Sox9 expression is elevated upon *Sox10* deletion in mouse and human melanoma cells [9], and critically contributes to the anti-tumorigenic effects observed upon *Sox10* inactivation in giant congenital naevus and melanoma.

Results

Expression analysis of SOXE factors in human skin melanocytes, giant congenital naevi, and primary melanoma *in vivo*

While SOX10 expression and function has been well established in adult melanocytes, naevi, and melanoma tissue in human and mice [9; 23], studies on SOX9 expression in melanocytic cells are controversial. SOX9 was reported to be expressed in cultured human melanocytes *in vitro* [32], human melanocytes *in vivo* [33], and in human melanoma [34-36]. Other reports, however, failed to reveal Sox9 mRNA and protein expression in melanoblasts and differentiated melanocytes during development and postnatally [21,37]. Given the close relationship between SoxE factors, one conceivable explanation for these discrepancies might be that antibodies raised against a given SoxE protein fail to discriminate between SOX9 and SOX10 epitopes. Indeed, when we performed immunohistochemistry on murine skin to test the specificity of various anti-SOX9 antibodies, several of them recognized both melanocytes and epithelial cells in the outer root sheet (a region in hair follicles known to express and functionally require Sox9; [37-38] (Supplemental Fig. 1). In contrast, the antibody sc-20095 exclusively detected protein expression in epithelial cells but not in melanocytes. Of note, in human melanoma cell lines *in vitro,* all antibodies tested but sc-20095 not only recognized SOX9, but also a protein of the molecular weight of SOX10 and detected by a SOX10-specific antibody (Supplemental Fig. 2A-D).

To further investigate the specificity of anti-SOX9 antibodies, we performed SOX10 knockdown in human melanoma cell lines *in vitro* and analyzed SOX10 and SOX9 expression using Western blot analysis (Supplemental Fig. 2E-K). As shown in Supplemental Fig. 2E-K, different anti-SOX9 antibodies used in earlier studies detected SOX10 protein expression, which was lost upon SOX10 knock-down. The only anti-SOX9 antibody, which did not display cross-reactivity with SOX10 protein, was sc-20095.

Therefore, we chose to reassess SOX9 expression in human melanocytes and melanocytic skin lesions using the specific SOX9 antibody sc-20095. Double immunostaining for SOX9 and MITF (Microphthalmia-associated transcription factor), an established marker of melanocytes [39] revealed no detectable SOX9 expression in human skin melanocytes *in vivo* (Fig. 1A-G). In contrast, SOX10 was readily detectable in human melanocytes (Supplemental Fig. 3A,B). Moreover, while SOX10 was expressed in 100% of human giant congenital naevi, SOX9 expression was not detected in the same set of patient samples (n=17; (Fig. 1H; Supplemental Fig. 3D); [9]). Likewise, all samples of a melanoma tissue microarray composed of 56 primary

melanoma biopsies revealed strong SOX10 expression (Fig. 1I; [9]). SOX9 expression, however, was found in only 41% (23/56) of the primary melanoma samples, in which it was expressed in a few scattered cells accounting for less than 10% of all melanoma cells (Fig. 1I). In contrast, expression of SOX9 was readily detectable in the epithelial lineage of normal skin as well as in basal cell carcinoma, an epithelial skin cancer (Fig. 1E, F; Supplemental Fig. 3C; [37,40]). To investigate the mRNA expression of SOX10 and SOX9 in a large set of human melanoma samples, we used of the TCGA (The Cancer Genome Atlas) database. Interestingly, the vast majority of human melanoma samples displayed much higher SOX10 than SOX9 expression (Fig. 1J) and only very few samples were characterized by a SOX9 high / SOX10 low expression pattern (Fig. 1K). Thus, SOX10 but not SOX9 is prominently expressed in normal human melanocytes, human giant congenital naevi, and primary melanoma.

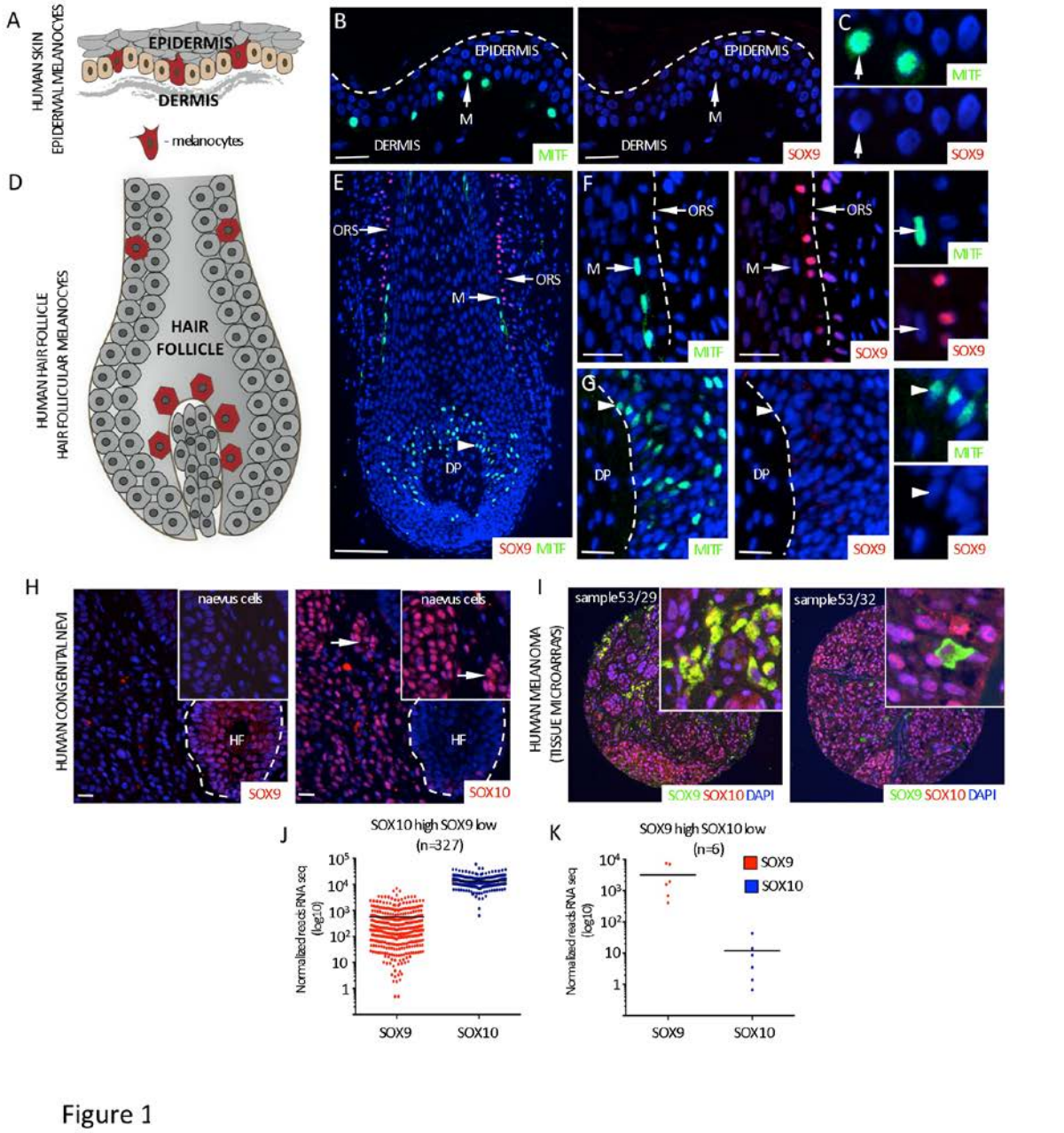

Figure 1

Figure 1. Differential expression of SOX10 and SOX9 in human melanocytes, human giant congenital naevi and human melanoma samples. A, Scheme showing the localization of epidermal melanocytes (in red) in the human skin. B, C, Immunostaining for MITF (green, left panel) and SOX9 (red, right panel) in the human skin demonstrating the lack of SOX9 expression in the epidermal melanocytes. Inserts show higher magnification images of MITF and SOX9 immunostainings. Scale bars, 25 µm. D, Scheme showing the localization of melanocytes (in red) within the hair follicle. E, Immunostaining for MITF (green) and SOX9 (red) in the human skin reveals the expression of SOX9 in the cells of outer root sheath but not in the MITF-positive melanoblasts/melanocytes. Scale bar 100 µm. F, G, High magnification images of immunostaining for MITF and SOX9 in the upper part of human hair follicle (F) and the follicular bulb (G). H, Analysis of SOX9 (red, left panel) and SOX10 (red, right panel)

expression in the patients with human giant congenital naevi demonstrates the lack of SOX9 expression in the SOX10-positive giant congenital naevi cells. Inserts show higher magnification. I, Representative examples of immunostaining for SOX9 (green) and SOX10 (red) in a tissue microarray of primary melanoma samples are shown. J-K, Distribution of SOX10 vs. SOX9 expression in human melanoma (based on TCGA database). 334 melanoma patients were divided in two groups, namely SOX10 High/ SOX9 Low and SOX10 Low / SOX9 high based on SOX10 and SOX9 expression levels. DP, dermal papilla; HF, hair follicle; M, melanocytes; ORS, outer root sheath. Scale bars, 25 µm.

SOX10 and SOX9 exhibit divergent functional roles in murine melanocyte stem cells and hair pigmentation

To corroborate our findings in an experimentally amenable system, we extended the analysis of Sox9 expression to mouse melanocytes, taking advantage of a previously described iDct-GFP mouse line (Fig.2A,B; [41]). Doxycycline-induced GFP-labelled melanocytes were isolated via fluorescence-activated cell sorting (FACS) and subjected to RNA-Seq analysis (Fig.2A). While Sox10, Mitf and Tyr were expressed at high levels (Sox10 reads were 1292, 1372, 1776 and 2488 at E15.5, E17.5, P1 and P7, respectively), the expression of Sox9 was extremely low (Sox9 reads were 68, 65, 105 and 128 at E15.5, E17.5, P1 and P7, respectively). These data are in accordance with earlier studies on Sox9 mRNA and protein expression in murine melanoblasts and melanocytes [21,37] and suggest that in contrast to Sox10, Sox9 expression is virtually absent in the melanocytic lineage during mouse embryogenesis and postnatally.

Melanocyte stem cells are found in a specialized region of the hair follicle called bulge and give rise to melanocyte progenitors and differentiated melanocytes [42]. The latter are located in the lower hair follicle portion termed bulb, where they transfer pigment to the growing hair. When melanocyte stem cells are functionally impaired, they fail to generate melanocytes, which results in hair graying [43]. To further investigate the expression of Sox10 and Sox9 in the melanocytic lineage of the mouse skin, we made use of *Dct::LacZ* transgenic mouse line expressing LacZ driven by the dopachrome tautomerase (Dct) promoter that allows genetic tracking of melanocyte stem cells and their derivatives in the hair follicle (Fig. 2C; [44]). Sox10 expression was detected in X-Gal-positive melanocyte stem cells located in the bulge region (Fig. 2E, upper panels) as well as in differentiated melanocytes in the hair follicular bulb (Fig. 2E, lower panels). Similarly to the situation in human melanocytes, immunostaining with the specific anti-Sox9 antibody sc-20095 demonstrated absence of Sox9 expression in X-Gal-positive melanocyte stem cells and their progeny (Fig. 2D, upper and lower panels). Sox9 expression was restricted to cells of the epithelial lineage, namely the outer root sheath and the epithelial stem cell compartment in the bulge area (Fig. 2D; Supplemental Fig. 4), in agreement with previous reports [37,38,45].

To address the function of *Sox10* and *Sox9* in the melanocytic lineage *in vivo*, we conditionally ablated either *Sox10* (Fig. 2H) or *Sox9* (Fig. 2F) using *Tyr-CreERT2* transgenic mice [46] carrying floxed alleles of *Sox10* [47] and *Sox9* [27], respectively. Tamoxifen-induced homozygous deletion of *Sox10* in *Sox10$^{fl/fl}$ Tyr-CreERT2* mice resulted in progressive hair graying, revealing an essential function of *Sox10* for melanocyte stem cell homeostasis (Fig. 2I, [23]). In contrast, homozygous deletion of *Sox9* in the melanocytic lineage did not cause hair graying even after a prolonged period after tamoxifen-induced gene

deletion (Fig. 2G). Thus, these two closely related genes are not only differentially expressed but also play distinct roles in the biology of melanocytes *in vivo*.

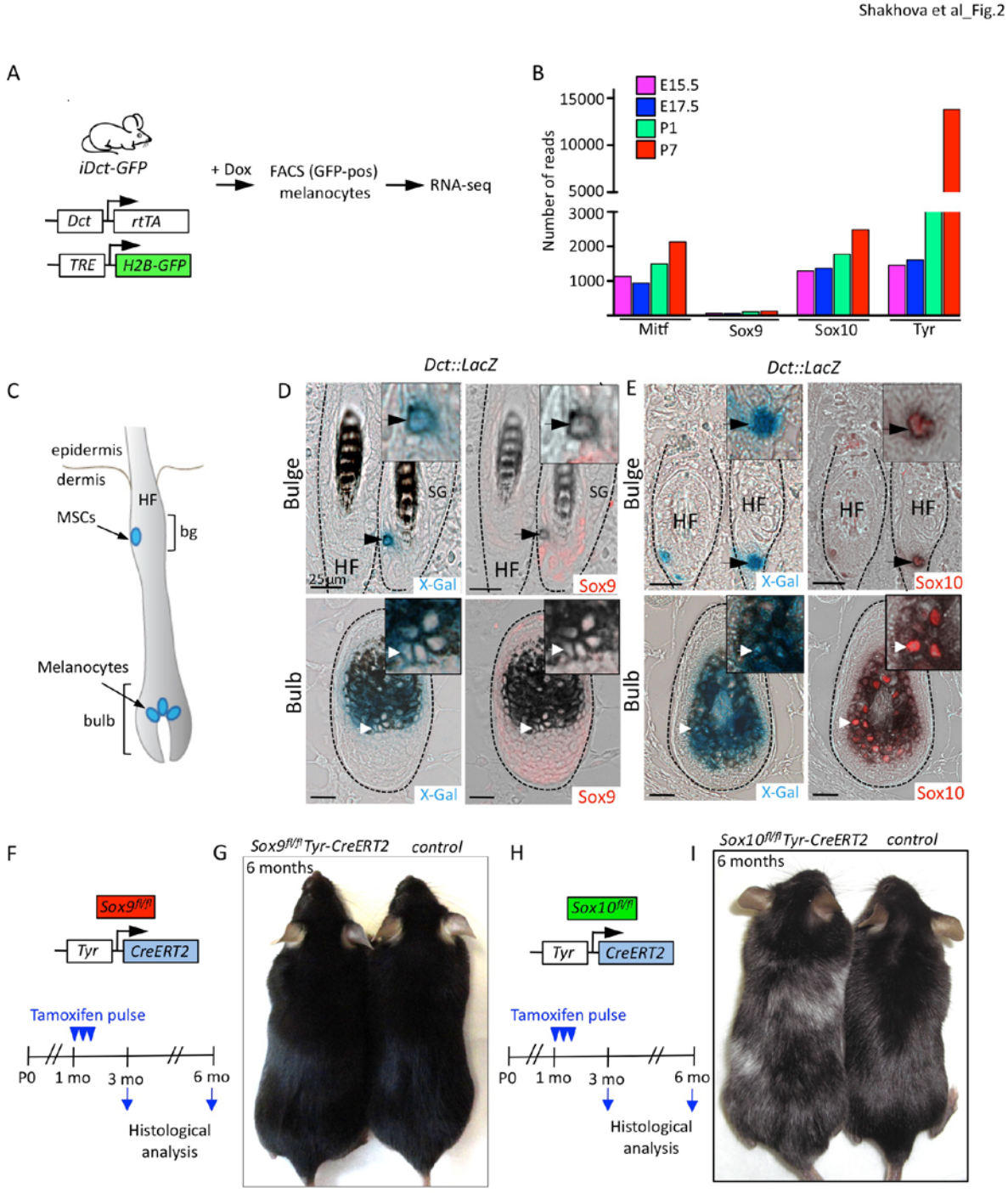

Figure 2

Figure 2. Sox9 is not expressed in the mouse melanocytic lineage and is not functionally required for the maintenance of melanocyte stem cells and melanocytes in the postnatal skin. A, A schematic representation of the experimental strategy used to analyze the expression of *Sox9*, *Sox10*, *Mitf* and *Tyr* genes in the melanocytic lineage *in vivo*. B, Results of RNA-seq analysis demonstrating high Sox10 and low Sox9 expression in melanocytic cells at various stages of development. C, A schematic representation of the anatomical location of the melanocyte stem cells, melanoblasts and differentiated melanocytes within the hair follicle in the mouse skin. D, X-Gal staining (blue) combined with Sox9 immunostaining (red) in skin sections of *Dct::LacZ* mice demonstrating the lack of the Sox9 expression in the melanocyte stem cells located in the bulge region of the hair follicle (upper panels) and in the differentiated melanocytes located in the hair follicular bulb (lower panels). E, Skin sections of *Dct::LacZ* mice stained for Sox10 (red) in combination with X-Gal staining (blue) reveal the expression of Sox10 in the melanocyte stem cells (upper panels) as well as in the differentiated melanocytes (lower panels). Dashed lines demarcate HFs. Insets show high magnification views. F-I, Experimental strategy used to analyze the effect of the lack of *Sox9* (F) and *Sox10* (H) expression in the mouse melanocytic lineage. Pictures of two representative mice at 6 months of age lacking *Sox9* gene (G) and *Sox10* gene (I) demonstrating the effects on hair graying. Bg, bulge; HF, hair follicle; MSCs, melanocyte stem cells; Mo, months; E 15.5, embryonic day 15.5; P0, postnatal day 0; SG, sebaceous gland. Scale bars, 25 μm.

Sox10 and Sox9 are differentially expressed in mouse giant congenital naevi and melanoma and exhibit functionally distinct roles in tumor initiation

To functionally assess the role of SoxE factors in melanomagenesis, we first performed immunohistochemical staining for Sox9 and Sox10 of giant congenital naevi formed in *Tyr::Nras^{Q61K}* mice and in melanoma derived from giant congenital naevi in *Tyr::Nras^{Q61K}Ink4a$^{-/-}$* mice [9,48]. In contrast to the widespread expression of Sox10 displayed by mouse naevi and melanoma tissue (Fig. 3B,D, [9]), immunostaining of both naevi and primary melanoma sections did not reveal any detectable expression of Sox9 protein (Fig. 3A,C; Supplemental Fig. 4B), consistent with the data that we have obtained for human giant congenital naevi and melanoma (Fig. 1H-I). Despite the lack of detectable Sox9 expression, low levels of Sox9 might be functionally implicated in the formation of melanocytic lesions arising in *Tyr::Nras^{Q61K}* mice. To address this issue, we generated *Tyr::Nras^{Q61K} Sox9$^{fl/fl}$ Tyr-CreERT2* mice and conditionally deleted both *Sox9* alleles by tamoxifen treatment of the mice (Fig. 3E). However, skin hyperpigmentation induced by oncogenic NRas was not affected in *Tyr::Nras^{Q61K} Sox9$^{fl/fl}$ Tyr-CreERT2* mice and was comparable to that presented by their control *Tyr::Nras^{Q61K}* littermates (Fig. 3F). These data reveal that in contrast to Sox10 (Fig. 3G,H}, Sox9 is not required for the formation of melanocytic lesions.

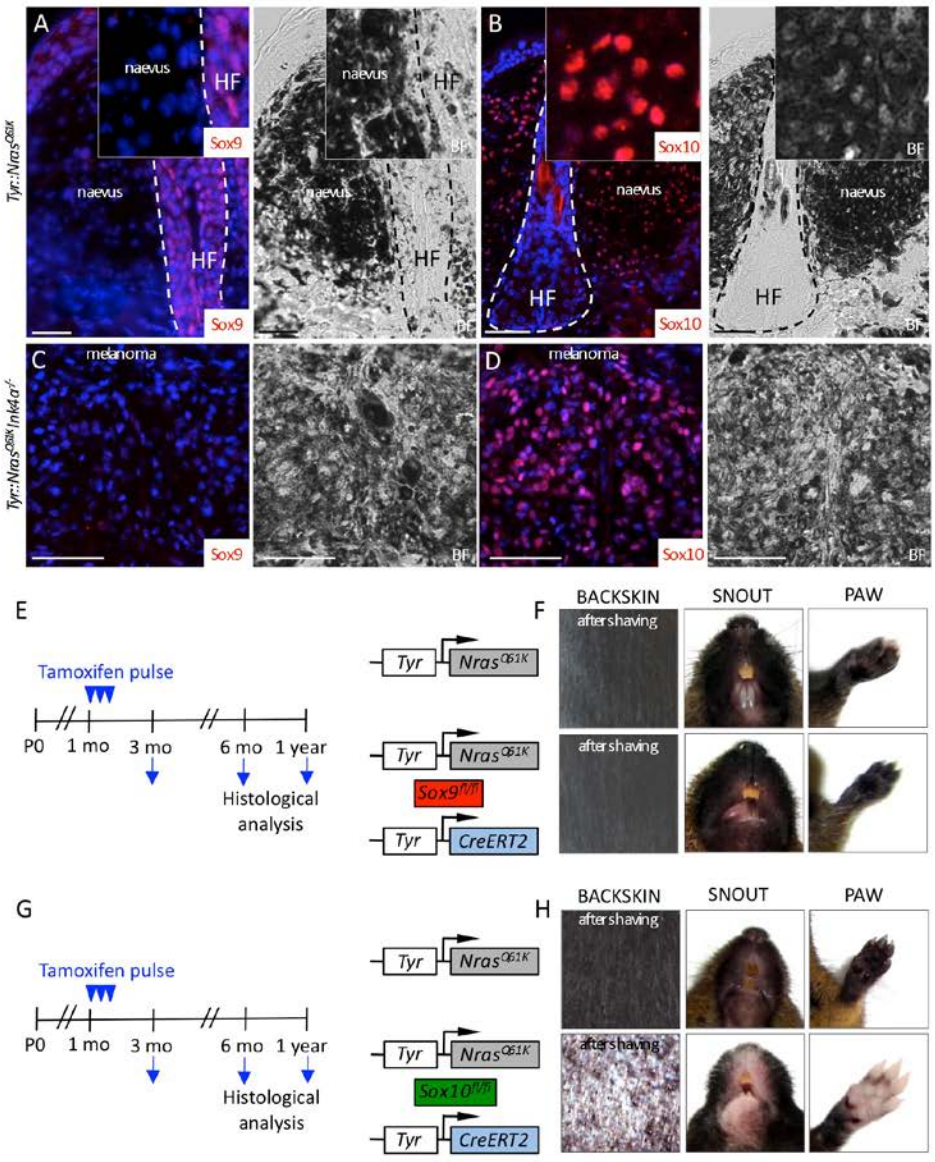

Figure 3

Figure 3. Mouse giant congenital naevi and melanoma reveal no expression of Sox9. A-D, Immunostaining for Sox9 (**A, C**) and Sox10 (**B, D**) in the skin sections of *Tyr::Nras*^{Q61K} and *Tyr::Nras*^{Q61K}*INK4a*$^{-/-}$ mice. **E-H**, Experimental strategy used to abrogate the expression of Sox9 (E) and Sox10 (G) in the mouse melanocytic lineage. Pictures of two representative mice 1 year after tamoxifen injections reveal no reduction in the skin hyperpigmentation in *Tyr::Nras*^{Q61K}*Sox9*$^{fl/+}$*Tyr-CreERT2* mice as compared to their *Tyr::Nras*^{Q61K} littermates (F) in contrast to a pronounced skin whitening observed upon Sox10 loss (H). BF, bright field; HF, hair follicle; mo, months; P0, postnatal day 0. Scale bars, 25 µm.

Deletion of SOX10 in giant congenital naevus cells results in the induction of SOX9 expression

To gain insight into the possible interplay between SOX10 and SOX9 during tumor progression, we quantified the expression levels of *SOX10* and *SOX9* in normal human melanocytes, in cells from giant congenital naevi, and in a melanoma cell line (M010817; [49]) using quantitative RT-PCR analysis (Fig. 4A-C). Notably, *NRAS*^{Q61K}-associated tumor progression was associated with an increase in *SOX10* expression (Fig. 4B). Cells from giant congenital naevi showed a 5-fold increase in *SOX10* expression when compared to normal melanocytes, while M010817 melanoma cells displayed a 10-fold increase in *SOX10* expression when compared to normal melanocytes (Fig. 4B). In striking contrast to the increase in *SOX10* expression, *SOX9* expression levels were low in human melanocytes and further decreased with melanoma progression (Fig. 4C).

To assess whether the disparate expression of SOX9 and SOX10 is a general feature of human melanoma samples, we analyzed the endogenous expression of these SOXE proteins in a large set of human melanoma cell lines previously categorized into cells with proliferative and invasive signatures, respectively [49]. Of note, all cell lines with a proliferative signature were characterized by high SOX10/low SOX9 mRNA expression (Fig. 4D). However, many cell lines with an invasive signature displayed the opposite expression pattern and showed low SOX10/high SOX9 mRNA expression. These date were confirmed on the protein level by Western blot analysis of several proliferative and invasive cell lines (Fig.4E).

Interestingly, an inverse correlation between *SOX10* and *SOX9* expression has previously been observed in several systems, including cultured human melanocytes, where upon the induction of differentiation, SOX10 levels were reduced concomitantly with an increase in SOX9 levels [32]. Thus, high expression of a given SoxE transcription factor might be causative for reduced expression of the related SoxE factor. We therefore asked whether deregulation of SOX10 leads to changes in SOX9 expression and found a significant upregulation of SOX9 mRNA expression upon SOX10 knockdown in human giant congenital naevus cells (Fig. 4F-H). This is in analogy to the upregulation of SOX9 mRNA previously observed in melanoma cells upon SOX10 knockdown [9]. Moreover, SOX10 knockdown also resulted in upregulation of SOX9 protein levels in human melanoma cells (Supplemental Fig. 5A). The combined data indicate that SOX10 normally suppresses SOX9 expression in cells from melanocytic lesions.

To address whether the findings in human cells *in vitro* also apply to the *in vivo* situation in mice, we isolated melanocytic progenitors using fluorescence-activated cell sorting (FACS) from the skin of *Tyr::Nras*^{Q61K} and *Tyr::Nras*^{Q61K} *Sox10*$^{LacZ/+}$ mice that lack one allele of *Sox10* (Fig. 4I) and subsequently measured Sox9 expression levels using quantitative RT-PCR (Fig. 4K, L). Elevated Sox10 levels mediated by oncogenic NRas in melanocytic cells from *Tyr::Nras*^{Q61K} mice [9] were associated with decreased Sox9 mRNA expression as compared to normal melanoblasts wild-type for NRas. However, reduced Sox10 levels brought about by Sox10 heterozygosity resulted in upregulation of Sox9 expression, as revealed by comparing cells from *Tyr::Nras*^{Q61K} with cells from *Tyr::Nras*^{Q61K} *Sox10*$^{LacZ/+}$ mice.

Thus, in cells derived from both human and mouse melanocytic lesions, reduced Sox10 levels were accompanied by elevated Sox9 expression.

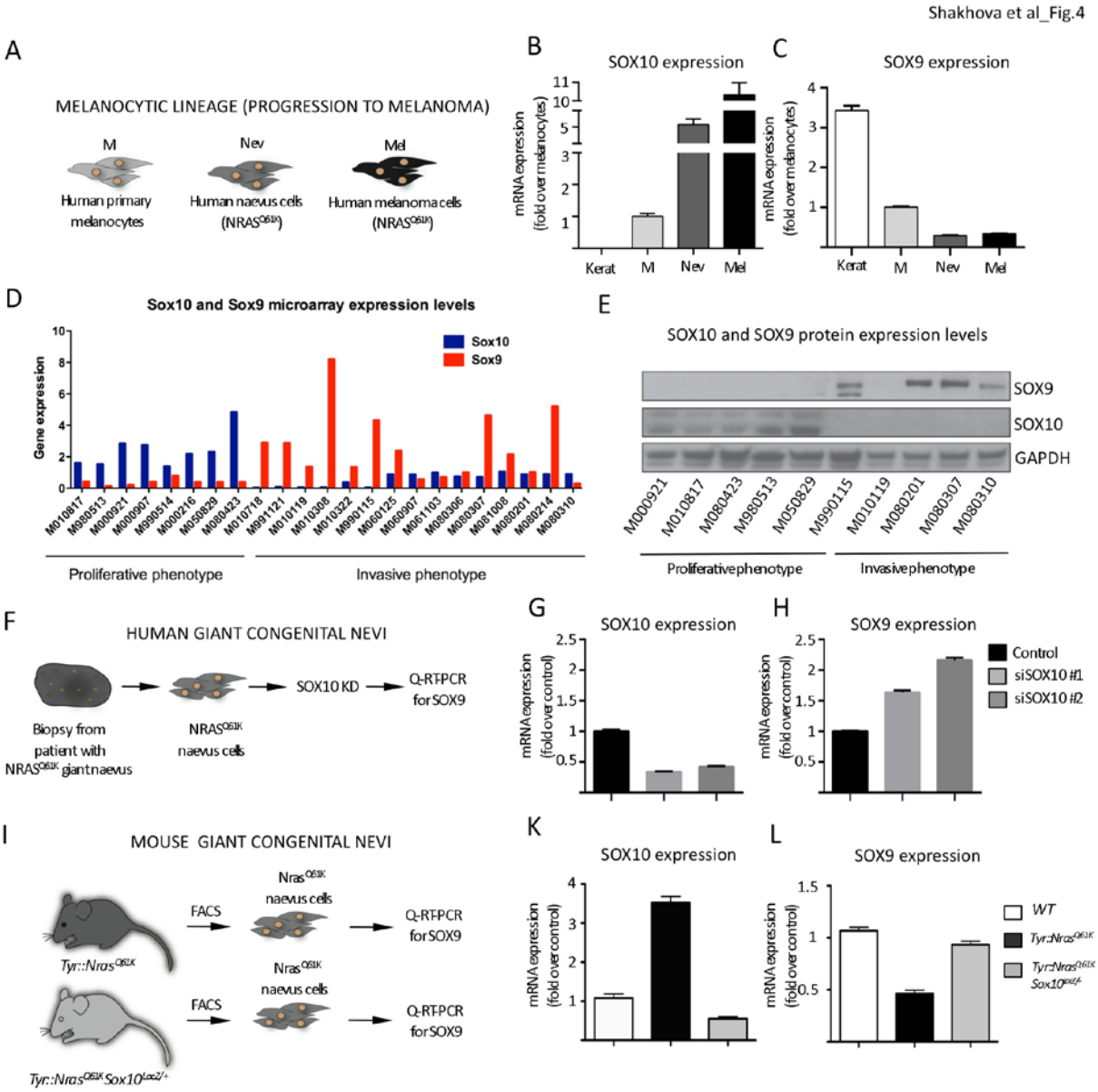

Figure 4

Figure 4. SOX10 knockdown results in elevated SOX9 expression in mouse and human melanocytes. A, Experimental design used to investigate the level of SOX9 and SOX10 expression *in vitro*. Cultured human keratinocytes, melanocytes, cells derived from biopsies of patients with giant congential naevi and melanoma cells (M010817 cell line) were subjected to RNA isolation and subsequent Q-RT-PCR analysis. Keratinocytes were used as a control. **B**, **C**, Quantitative real-time PCR analysis showing the decline of SOX9 expression (**C**) and increase of SOX10 expression (**B**) that

correlate with the acquisition of malignant state by human NRAS^{Q61K}-mutated cells. Giant congenital naevi and melanoma cells were sequenced to confirm *NRAS^{Q61K}* mutation (data not shown). Data are presented as the mean fold change and are normalized over levels found in melanocytes. **D**, **E**, SOX10 and SOX9 expression in a large set of proliferative and invasive cell lines analysed by gene expression using microarrays (**D**) and Western blot (**E**) techniques. **F,** Experimental design used to deregulate SOX10 expression in human cells derived from the biopsy of a patient with NRASQ61K---mutated giant congenital naevus. **G, H**, Quantitative real---time PCR analysis of SOX10 (G) and SOX9 (H) expression after the knockdown of SOX10. I, Experimental design used to analyze the expression of Sox9 in the melanocytic lineage from Tyr::NrasQ61K and Tyr::NrasQ61K Sox10LacZ/+ mice. K, L, Cells were isolated from the trunk skin of Tyr::NrasQ61K and Tyr::NrasQ61K Sox10LacZ/+ mice and stained for Melan---a and c---Kit antibodies. FACS---sorted cells were subsequently used for the RNA isolation and quantitative real---time PCR with primers specific for the coding regions of Sox9 gene. Data are presented as the mean fold change and are normalized to the control. Kerat, keratinocytes; M, melanocytes; Nev, naevus cells; Mel, melanoma cells; KD, knock down.

SOX10 and SOX9 display antagonistic functions in melanoma cells

Based on our findings it is conceivable that increased levels of SOX9 might mediate at least some of the anti-tumorigenic effects observed upon SOX10 loss-of-function in melanoma. To address this hypothesis, we overexpressed SOX9 in human M010817 melanoma cells *in vitro* and compared the gene expression profile of these cells with that of *SOX10* knock-down melanoma cells, using the parental M010817 cell line as control [9]. Unsupervised hierarchical clustering revealed that overexpression of *SOX9* led to a gene expression profile closely resembling the *SOX10* knockdown-signature, which included in both conditions regulators of cell cycle progression, apoptosis, and melanocytic and mesectodermal differentiation (Fig. 5A). Among others, these data suggest that while SOX10 acts as an inhibitor of apoptosis in melanoma cells, SOX9 elicits a proapoptotic response. Similarly, SOX10 and SOX9 appear to play antagonistic functions in the regulation of the cell cycle, melanocytic differentiation, and mesectodermal differentiation (Fig. 5A).

Notably, *SOX9* overexpression resulted in decreased expression of a number of genes associated with melanocytic differentiation, such as MLANA, MITF, DCT, TYR, and importantly SOX10. To confirm the downregulation of SOX10 upon SOX9 overexpression observed in microarray analysis (Fig. 5A) also on the protein level, we performed Western blot analysis and observed a pronounced downregulation of SOX10 protein upon SOX9 overexpression in human melanoma cell lines (Fig. 5B). Moreover, chromatin immunoprecipitation assays in human melanoma M010817 cells indicated that SOX9 binds to the promoter of SOX10, suggesting a direct regulation of the *SOX10* gene by SOX9 (Fig. 5C). Thus, whereas SOX10 loss-of-function leads to increased SOX9 expression (Fig. 4), high levels of SOX9 suppress SOX10 expression, revealing cross-regulatory interactions between these two transcription factors.

Next, we addressed whether the cross-regulation between SOX10 and SOX9 is functionally relevant in human melanoma cells. To this end, we performed RNAi experiments to test whether interfering with SOX9 overexpression upon SOX10 knockdown could rescue M010817 melanoma cells (Fig. 5D-F). Using two independent sets of siRNAs, the elevated SOX9 levels observed in SOX10 knockdown cells were reverted below control levels by means of

concomitant SOX9 knockdown (Fig. 5E). Importantly, both SOX9 gain-of-function and SOX10 knockdown promoted apoptosis (Fig. 5F; Supplemental Fig. 5B). However, SOX10 knockdown cells were rescued when SOX9 expression was simultaneously downregulated, resulting in numbers of apoptotic cells comparable to those found in control cells (number of Annexin V-positive apoptotic M010817 cells: control, 9.95±0.9%; SOX10 siRNA #1, 19.96±0.13%; SOX10 siRNA #2 18.8±0.49%; combination of SOX10 siRNA #1/SOX9 siRNA, 9.45±0.79%; combination of SOX10 siRNA #2/SOX9 siRNA. 10.75±0.4%) (Fig.5F; Supplemental Fig. 5B). These data indicate that at least *in vitro*, SOX9 plays a key role in mediating the cellular phenotype obtained in human melanoma cells upon suppression of SOX10.

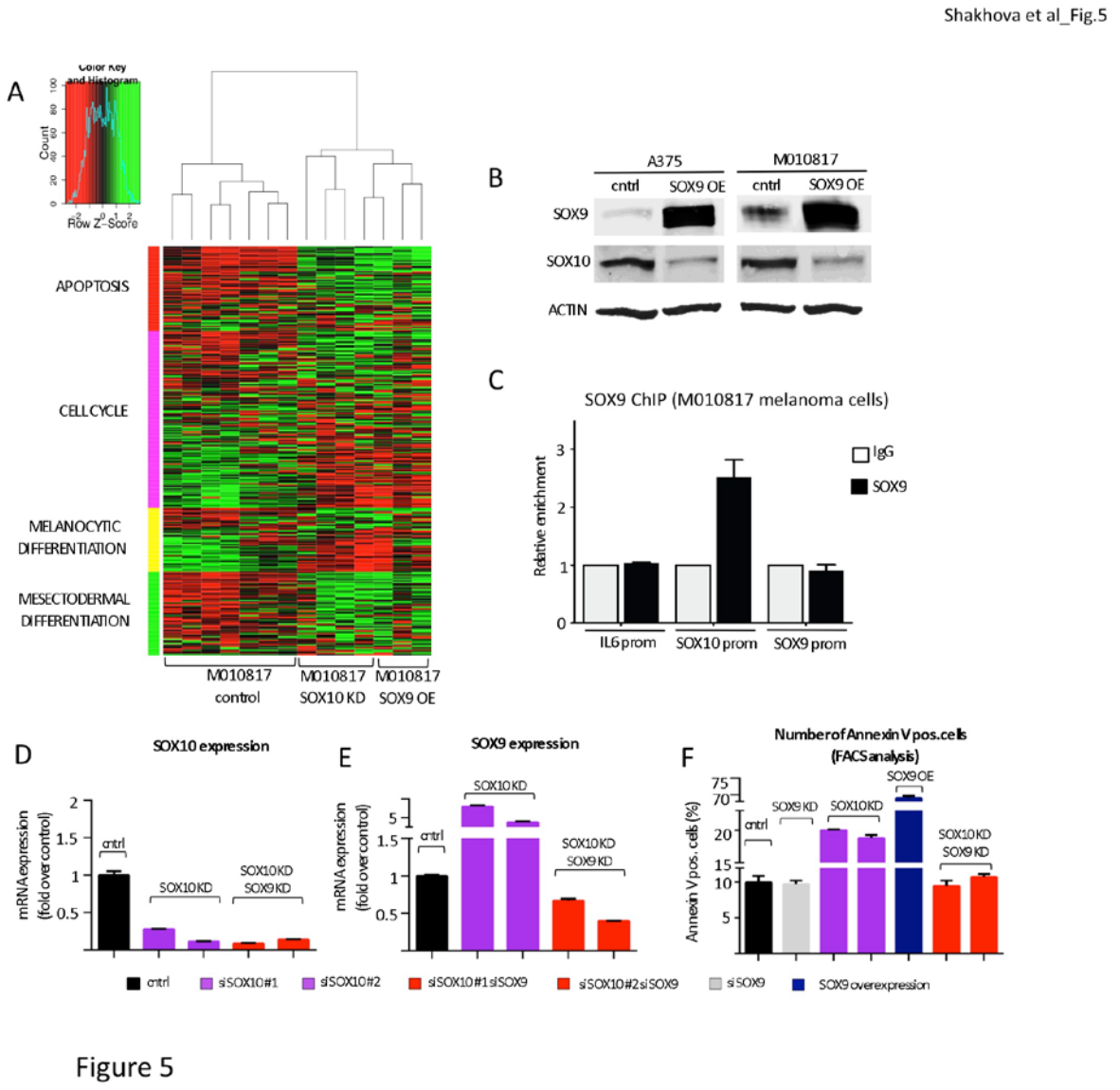

Figure 5

Figure 5. Experimental suppression of SOX9 expression rescues the effects of SOX10 downregulation in human melanoma cells. A, SOX9 overexpression in human melanoma cells closely

resembles the gene expression signature of SOX10 knockdown as revealed by unsupervised hierarchical clustering of control M010817 melanoma cells, SOX9 overexpressing M010817 cells and SOX10 knock down M010817 cells. Microarray gene expression accession number: GSE37059. **B**, Western blot analysis showing that SOX10 expression is downregulated upon overexpression of SOX9 in two independent human melanoma cell lines (A375 and M010817). **C**, Chromatin immunoprecipitation assay demonstrating the binding of SOX9 to the promoter of SOX10 in human melanoma M010817 cells. **D, E,** Quantitative real-time PCR analysis of SOX10 (**E**) and SOX9 (**F**) expression after the knockdown of SOX10 and after the double knockdown of SOX10 and SOX9 in M010817 cell line. **F**, Quantification of number of Annexin V-positive cells based on the FACS analysis in the melanoma M010817 cells upon SOX9 KD, SOX10 KD or double SOX9/SOX10 KD. OE, overexpression; KD, knock down; ChIP, chromatin immunoprecipitation; prom, promoter.

Deletion of Sox9 rescues the phenotype of Tyr::Nras^{Q61K}Sox10$^{fl/+}$Tyr-CreERT2 mice, restoring Nras^{Q61K}-mediated naevus formation

As in human melanoma cells *in vitro*, reducing Sox10 levels *in vivo* elicits an anti-tumorigenic effect, preventing melanocytic hyperplasia in *Tyr::Nras^{Q61K}Sox10$^{fl/+}$Tyr-CreERT2* mice [9]. To functionally test whether upregulation of Sox9 is the key factor accountable for the lack of hyperplasia in these mice *in vivo*, we performed simultaneous conditional ablation of both *Sox10* and *Sox9* genes in the *Tyr::Nras^{Q61K}* mice (Fig. 6A, B). In agreement with our previous observations [9], the skin of the snout, paws and the back was noticeably lighter in color in *Tyr::Nras^{Q61K}Sox10$^{fl/+}$Tyr-CreERT2* mice as compared to their control *Tyr::Nras^{Q61K}* littermates (Fig. 6A, left and middle panels). In contrast, macroscopic examination of the skin of *Tyr::Nras^{Q61K}Sox10$^{fl/+}$Sox9$^{fl/fl}$Tyr-CreERT2* animals showed pronounced hyperpigmentation, a hallmark of the skin phenotype found in *Tyr::Nras^{Q61K}* mice (Fig. 6A, right panels). Thus, conditional deletion of *Sox9* rescued the effect of *Sox10* haploinsufficiency in *Tyr::Nras^{Q61K}* mice.

As shown in Figure 2, conditional ablation of both alleles of *Sox10* in the normal melanocytic lineage using *Tyr-CreERT2* resulted in loss of functional melanocytes and hair graying (Fig. 2I). Likewise, homozygous deletion of *Sox10* in *Tyr::Nras^{Q61K}* mice not only prevented *Nras^{Q61K}*-stimulated skin hyperpigmentation, but also led to hair greying in these mice (Fig. 6B, left panels). To determine whether these phenotypes involve Sox9 activity and whether, therefore, loss of *Sox9* could rescue the effects of homozygous *Sox10* deletion, we generated *Tyr::Nras^{Q61K}Sox10$^{fl/fl}$Sox9$^{fl/fl}$Tyr-CreERT2* animals. Surprisingly, loss of *Sox9* in *Sox10* homozygous conditional knock-out animals efficiently restored skin hyperpigmentation as seen on the snout and paws of *Tyr::Nras^{Q61K}Sox10$^{fl/fl}$Sox9$^{fl/fl}$Tyr-CreERT2* animals when compared to mice lacking *Sox10* but not *Sox9* in the melanocytic lineage (Fig. 6B, right panels).

We have previously demonstrated that skin hyperpigmentation in *Tyr::Nras^{Q61K}* mice is due to hyperplasia of ectopically located pigment cells emerging in the dermis, around the upper part of the hair follicle [9]. To measure the degree of hyperpigmentation in *Tyr::Nras^{Q61K}* mice with conditional *Sox10* deletion (heterozygous and homozygous) versus mice simultaneously lacking both *Sox9* alleles and one or both *Sox10* alleles in the melanocytic lineage, we quantified the percentage of hair follicles associated with ectopically located melanocytic cells

in the back skin of these mouse lines. In *Tyr::Nras*^{Q61K} mice, more than 90% of all hair follicles displayed ectopic pigment cells (Fig. 6D). In contrast, in the absence of one or both alleles of *Sox10*, there were almost no hair follicles with ectopic pigment cells, despite Nras^{Q61K} expression (Fig. 6C, D). Strikingly, however, the percentage of hair follicles associated with ectopic melanoblasts in *Tyr::Nras*^{Q61K}*Sox10*$^{fl/+}$*Sox9*$^{fl/fl}$*Tyr-CreERT2* animals was reverted to numbers similar to those found in *Tyr::Nras*^{Q61K} mice (93±1.8% and 96±1%, respectively) (Fig. 6C, D). Moreover, even in the absence of both *Sox10* alleles, loss of *Sox9* rescued the Nras^{Q61K}–dependent appearance of melanocytic cells found outside hair follicles (93.5±3%) (Fig. 6D). These data reveal a key role of Sox9 in preventing melanoma initiation and provide novel insights into the functional interplay between Sox10 and Sox9 during melanoma formation.

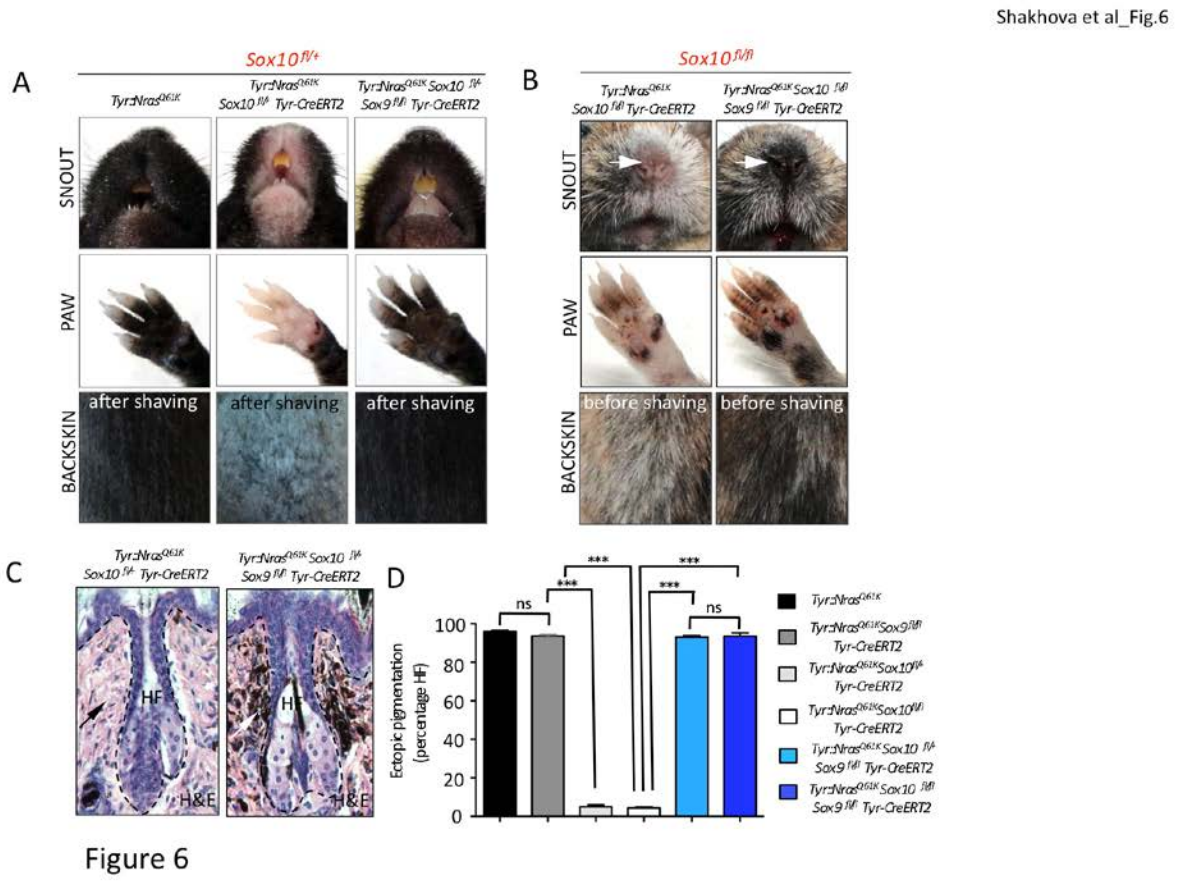

Figure 6

Figure 6. Homozygous deletion of *Sox9* rescues the effects of Sox10 loss in *Tyr::Nras*^{Q61K} mice and restores hyperpigmentation *in vivo*. A, B, Representative pictures of back skin, paws and snouts from mice of the indicated genotypes. C, D, Histological evaluation of the hyperpigmentation phenotype in the skin. Haematoxylin and eosin staining of back skin (C) was followed by the quantification (D) of the percentage of hair follicles associated with the hyperpigmentation. HF, hair follicle; H&E, haematoxylin and eosin.

Discussion

Our study identifies the structurally related transcription factors SOX10 and SOX9 as functionally antagonistic regulators of postnatal melanocyte and melanoma development. Although we did not find SOX9 to be expressed in the melanocytic lineage when SOX10 is present, SOX9 expression becomes evident upon SOX10 inactivation in naevus and melanoma cells. In this context, SOX9 appears to promote the major cellular processes induced by SOX10 loss-of-function, namely stop of proliferation and apoptosis. Intriguingly, SOX9 and SOX10 are engaged in a cross-regulatory feedback loop whereby SOX9, which is induced upon SOX10 inactivation, itself suppresses SOX10, thus strengthening an anti-tumorigenic program.

In many cell lineages and tissues, SOX10 and SOX9 are co-expressed and functionally redundant [50]. We propose that this is not the case in melanocytic cells and that SOX9, unlike SOX10, is neither required for normal melanocyte stem cell homeostasis nor for formation of congenital nevi and primary melanoma. Our findings disagree with some previously published studies reporting SOX9 expression in the normal and tumor-associated melanocytic lineage [32-34,36,51]. However, as we demonstrate here, most previously used anti-SOX9 antibodies display cross-reactivity with SOX10, owing to the close relationship between these two SoxE factors. Having identified anti-SOX10 and anti-SOX9 specific antibodies, we reveal virtually exclusive expression patterns of these transcription factors in the normal human skin and in a large set of melanoma biopsies and cell lines. While SOX10 expression is restricted to neural crest derivatives, including melanoblasts, differentiated melanocytes, and virtually all human naevus and melanoma biopsies tested (Fig. 1; Supplemental Fig. 3; [9], SOX9 expression in melanocytic cells was restricted to few scattered cells in a subset of melanoma biopsies. In contrast, SOX9 was strongly expressed in epithelial cells of the hair follicle, which are devoid of SOX10 expression.

In support of these data, Sox10 protein expression in the mouse skin is detected *in vivo* throughout all stages of melanocyte development from stem cells to differentiated melanocytes in the hair follicular bulb (Fig. 2). Mice lacking *Sox10* in the melanocyte lineage display hair graying, indicating that Sox10 is necessary for maintenance of melanocyte stem cells and committed melanoblasts [23] (Fig. 2). Likewise, Sox10 is required for the establishment of giant congenital naevi as well as melanoma [9]. In contrast, murine Sox9 appeared not to be expressed in melanocytic cells of the normal skin, nevi, and primary melanoma, while it was readily detectable in epithelial cells in accordance with previous reports [33,37,38]. Importantly, loss of function analyses failed to reveal a crucial role of *Sox9* in normal melanocytes, as conditional deletion of *Sox9* did not affect generation and long-term maintenance of melanocytes *in vivo* and did not result in hair graying, a phenotype characteristic for the loss of *Sox10*. Likewise, lack of *Sox9* did not prevent emergence of melanocytic lesions induced by oncogenic $NRas^{Q61K}$ (Fig. 3). These data demonstrate that Sox10 and Sox9 are not only expressed in different cellular compartments in the skin, but also play distinct roles in normal and transformed melanocytes.

In cell types other than melanocytes Sox9 and Sox10 can act redundantly. For instance, in oligodendroglial progenitors, concomitant expression of Sox9 can compensate for the loss of

Sox10 [52,53]. Similarly, in avian and *Xenopus* embryos, Sox9 and Sox10 are co-expressed in premigratory neural crest cells and are both able to induce ectopic neural crest cell formation upon forced expression in chicken neural tube [11,24,54]. In addition, the two factors are able to cross-regulate each other at this early stage of neural crest formation. Interfering with Sox10 function leads to inhibition of Sox9 expression [55], suggesting that Sox10 is required for the expression of Sox9 in pre-migratory neural crest. On the other hand, Sox9 overexpression in *Xenopus* embryos leads to upregulation of Sox10 expression [24], suggesting that Sox9 can also act upstream of Sox10. As development proceeds, however, Sox10 expression persists in the trunk neural crest and is downregulated in cranial neural crest cells giving rise to mesectodermal structures, while Sox9 expression is absent in trunk neural crest cells but present in the cranial neural crest [24,26]. These divergent expression patterns are established by signaling pathways differentially regulating transcription of Sox9 and Sox10, respectively. In particular, TGFβ (transforming growth factor β) simultaneously triggers induction of Sox9 and reduction of Sox10 expression [56]. Accordingly, mice lacking *Sox10* display phenotypes that are distinct from those obtained upon loss of *Sox9* [13,54,56-58]. In particular, Sox10 but not Sox9 is expressed in and required for the generation of melanoblasts during mouse embryogenesis [13,21]. Finally, in agreement with our expression studies on human skin, humans carrying mutations in *SOX9* display campomelic dysplasia affecting the skeleton and reproductive system but not melanocytes, whereas patients with mutations in *SOX10* often exhibit pigmentary anomalies [20,50,59].

However, the divergent functions of Sox10 and Sox9 in the skin appear not to be simply due to their differential expression patterns. Depending on the cellular context, these two transcription factors can also elicit different responses in one and the same cell lineage rather than playing redundant roles. Studies in *Xenopus* embryos demonstrated that while expression of Sox10 at the two-cell stage was sufficient to activate the expression of Trp-2 (Dct) and the induction of melanocytic precursors, the expression of Sox9 failed to do so [24]. In mice, loss of Sox9 promotes apoptosis and other phenotypes in neural crest cells, but Sox10 is maintained in these cells and cannot rescue the *Sox9*-mutant phenotype [54]. Moreover, while Sox9 activates the expression of genes involved in the induction of osteochondrogenesis in neural crest cells in pharyngeal arches [26,28,60], Sox10 is involved in the specification of a glial and melanocytic gene expression program [13]. In this context, Sox10 and Sox9 play antagonistic roles, in that Sox9 promotes cells cycle exit and mesenchymal fates, while Sox10 activates proliferation and suppresses mesenchymal fate acquisition [56]. Accordingly, *Sox10* inactivation results in induction of Sox9-dependent fates in postmigratory neural crest cells. This interplay between Sox10 and Sox9 functions during normal neural crest development is highly reminiscent of our findings in melanocytic lesions, where Sox10 also promotes proliferation and survival, while Sox9 counteracts these cellular processes. Indeed, in human melanoma cells, loss of SOX10 not only resulted in upregulation of SOX9 expression, but also in global transcriptional changes highly similar to the changes observed upon SOX9 overexpression (Fig. 5), indicating that these factors appear to play opposing functions in melanoma. Interestingly, a study by Passeron et al. revealed that overexpression of SOX9 prevents melanoma formation [35] by increasing the expression of the CDK inhibitor p21 and subsequent cell cycle arrest. Likewise, a recent report by Pavan and colleagues established

that the expression of p21 and p27 were increased upon SOX10 knockdown [31]. Thus, our data might provide one explanation for the anti-tumorigenic effect of SOX9, namely by downregulation of SOX10. This is in accordance with our previously published results on the essential role of SOX10 for melanoma initiation and progression [9]. Of note, the anti-tumorigenic effect elicited by suppressing SOX10 was abolished by concomitant SOX9 inactivation both in human melanoma cells as well as in mice. Thus, antagonistic SOX10/SOX9 constitutes a key node in the genetic network underlying melanomagenesis. Nonetheless, it is conceivable that further cues mediate SOX10-pro- and SOX9-anti-tumorigenic effects, respectively. Moreover, our data do not exclude a role of SOX9 at later stages of melanoma disease progression, in particular during metastasis formation by invasive cells. Indeed, while we could attribute a SOX10 high/SOX9 low signature to proliferative human melanoma cell lines and to all human and murine melanoma tissues analyzed, several human melanoma cell lines reported to display invasive features [49] exhibited SOX10 low/SOX9 high expression. Although this remains to be shown, these invasive cell lines with SOX10 low/SOX9 high expression might have been established by capturing or inducing invasive tumor cells that appear to be rather rare in biopsies of bulk tumor tissue. Likewise, apart from experimentally reducing SOX10 levels, other stimuli such as UV exposure might also lead to upregulation of SOX9 [51]. In any case, our discovery of the antagonistic interaction between SOX10 and SOX9, together with the further characterization of their mode of action in melanoma cells, might not only provide new mechanistic insights into how SoxE group proteins are regulated and act in the context of melanoma initiation and maintenance, but might also point to novel strategies for melanoma therapies.

Materials And Methods

Human Specimens

All analyses involving human skin, giant congenital naevi and melanoma tissue were performed in accordance with the ethical committee in canton Zurich, Switzerland. TMA containing melanoma tissue was constructed as previously described [61].

Mice

Tyr::Nras^{Q61K} [48] were provided by F. Beermann (EPFL Lausanne, Switzerland). *Dct-LacZ* mice were described previously [44]. *Sox10*$^{fl/fl}$ mice were described previously [47]. *Sox9*$^{fl/fl}$ mice [27] were a kind gift from G. Scherer (Institute of Human Genetics, Freiburg, Germany). *Tyr-CreERT2* line [46] was provided by L. Chin (The University of Texas MD Anderson Cancer Center, Houston, Texas, USA). *Rosa26-lacZ* mice were obtained from Jackson laboratory. All animal experiments were performed in accordance with Swiss law and have been approved by the veterinary authorities of Zurich.

Tamoxifen injections

Mice were subjected to intraperitoneal injections of tamoxifen (T5648, Sigma), diluted with the mixture of ethanol and sunflower oil (1:9 ratio). Tamoxifen was injected for 5 consecutive days.

Histological analysis and immunohistochemistry

Immunohistochemistry on paraffin sections was performed as previously described [9]. Briefly, skin samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin. For immunohistochemistry, antigen retrieval was performed in citrate buffer (pH 6.0) for 10 minutes at 110°C in HistoPro (Rapid Microwave Histoprocessor, Milestone, USA). The following primary antibodies were used: anti-Sox10 (goat, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Sox10 (mouse, 1:200, R&D), anti-Sox9 (rabbit, 1:100, sc-20095, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Sox9 (rabbit, 1:100, ab36748, Abcam), anti-Sox9 (M00006662, Abnova), anti-Sox9 (AB5535, Millipore), anti-Sox9 (GTX 109661, GenTex), anti-MITF (mouse, clone 6D3, 1:500) was a kind gift from Heinz Arnheiter (NIH, USA). Images were captured with a Leica DMI 6000B Microscope and using LAS AF (Leica Application Suite Advanced Fluorescence) software. For whole mount X-Gal staining, skin samples were fixed with 4% buffered paraformaldehyde, washed with PBS and subjected to X-Gal staining solution overnight at 37°C. After several washing steps, tissue was paraffin embedded and sectioned. 5 µm thick sections were further counterstained with eosin solution and mounted.

RNA isolation, reverse transcription and quantitative PCR.

Total RNA was isolated using Trizol according to manufacturer's instructions (Invitrogen). 1 µg aliquots of RNA were reverse transcribed with Reverse Transcription System (Promega) according to the manufacturer's instructions. Data collection and analysis were performed by ABI Viia7 Fast Real-Time PCR Systems (Applied Biosystems). Gene expression values of averaged triplicate reactions were normalized to *RPL28* expression levels. RPL28 primers are as follows: 5'-GCAATTGGTTCCGCTACAAC-3' and 5'-TGTTCTTGCGGATCATGTGT-3'. The expression of SOX10 and SOX9 was measured using primers purchased from QIAGEN: SOX10 (Hs_SOX10_1_SG); SOX9 (Hs_SOX9_1_SG).

Sequencing

Cells derived from patients with giant congenital naevi were sequenced for NRAS. Primers for sequencing for Exon 1 (mutation G12) and Exon 2 (mutation Q61K) of NRAS gene were as follows: NRAS_1F 5'-ATAGAAAGCTTTAAAGTACTG-3' and NRAS_1R 5'-TTCCTTTAATACAGAATATGG-3', NRAS_2F 5'-CCCCTTACCCTCCACAC-3' and NRAS_2R 5'-AACCTAAAACCAACTCTTCCCA-3'.

Cell culture and transfection assays

Silencing RNA (siRNA) transfection was carried out using INTERFERin transfection solution according to the manufacturer's protocol (Polyplus-transfection, Illkirch, France). Cells were transfected with 10 nM of siRNA (Qiagen) for 96 hours before RNA was extracted or used for FACS analysis. As control siRNA, the All-Star negative siRNA sequence (Qiagen) was used, and gene-specific siRNAs targeting siSOX10 (SI00729414, SI00729421) and siSOX9

(SI00007595, SI00007609) were obtained from Qiagen. Transfection of DNA was carried using JetPEI transfection solution according to the manufacturer's protocol (Polyplus-transfection, Illkirch, France). Cells were transfected with 1 ug of pCMV6-SOX9 (Origene SC321884) or empty vector for 96 hours before RNA was extracted or used for FACS analysis.

Melanocyte FACS and RNA seq

Melanocytes were purified by FACS from doxycycline-treated iDct-GFP mice as previously described [41]. Total RNA was prepared from FACS-sorted cell fractions containing GFP-positive melanoblasts/melanocytes according to standard Illumina RNA-Seq paired-end protocol and sequenced on the Illumina GAIIx to 80 bp per read.

Microarray analysis

Total RNA was isolated from melanoma cell cultures using TRIzol according to the manufacturer's instructions (Invitrogen). Total RNA was amplified and biotin-labelled using the Message Amp II-Biotin Enhanced aRNA Amplification Kit (Ambion, Austin, TX, USA). Biotin-labelled RNA was hybridized to Affymetrix HG-U133 plus 2.0 oligonucleotide microarrays following the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). After hybridization, microarrays were washed and stained using a GeneChip Fluidics Station 450 (Affymetrix) and then scanned using a GeneChip Scanner 7G (Affymetrix). Raw data was processed by R using the affycoretools package (RMA). Gene expression datasets for SOX10 knockdown were obtained from NCBI GEO GSE37059. Gene expression analysis was performed by R using the limma package. P-values were adjusted by FDR p-value adjustment. For melanoma cell lines analysis (proliferative vs invasive): Normalized expression values were downloaded from GSE4840 containing microarray data for twenty three melanoma cell cultures. Pearson's product moment correlation (r) was calculated for the SOX10 andSOX9 expression values across all twenty three samples. P-value was determined from the t statistic calculated from r.

Flow cytometry and cell sorting

Skin tissue (from back skin) was digested with a mixture of Dispase (Roche) and Collagenase I (Worthington) for 1 hour at 37°C and enzymatic reaction was stopped by addition of DMEM media supplemented with 10%FCS as previously described [9]. Subsequently, single cell suspension was filtered through 40 µm strainers (BD). For cell cycle analysis, Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit (Invitrogen) was used. Cells were labeled with PI according to manufacturer's protocol and DNA content was measured using a BD FACSCanto II flow cytometer (BD Biosciences) and a BD FACSDiva software (BD Biosciences). For measurement of apoptosis, Annexin V-PE Apoptosis Detection Kit I (BD Pharmingen, 559763) was used. FACSAria sorter and FACS DiVa software (BD Biosciences) were used for cell sorting.

Chromatin Immunoprecipitation

ChIP analysis was performed as previously described [62]. Sox9 antibody was from Santa Cruz Biotechnology (sc-20095, Santa Cruz Biotechnology). SOX10 promoter sequences were amplified with forward primer (5'-CCTCTGCCTCGTGTGACTAC-3') and reverse primer (5'-TCCTGTCTGGAGTGGGCTG-3').

Acknowledgments

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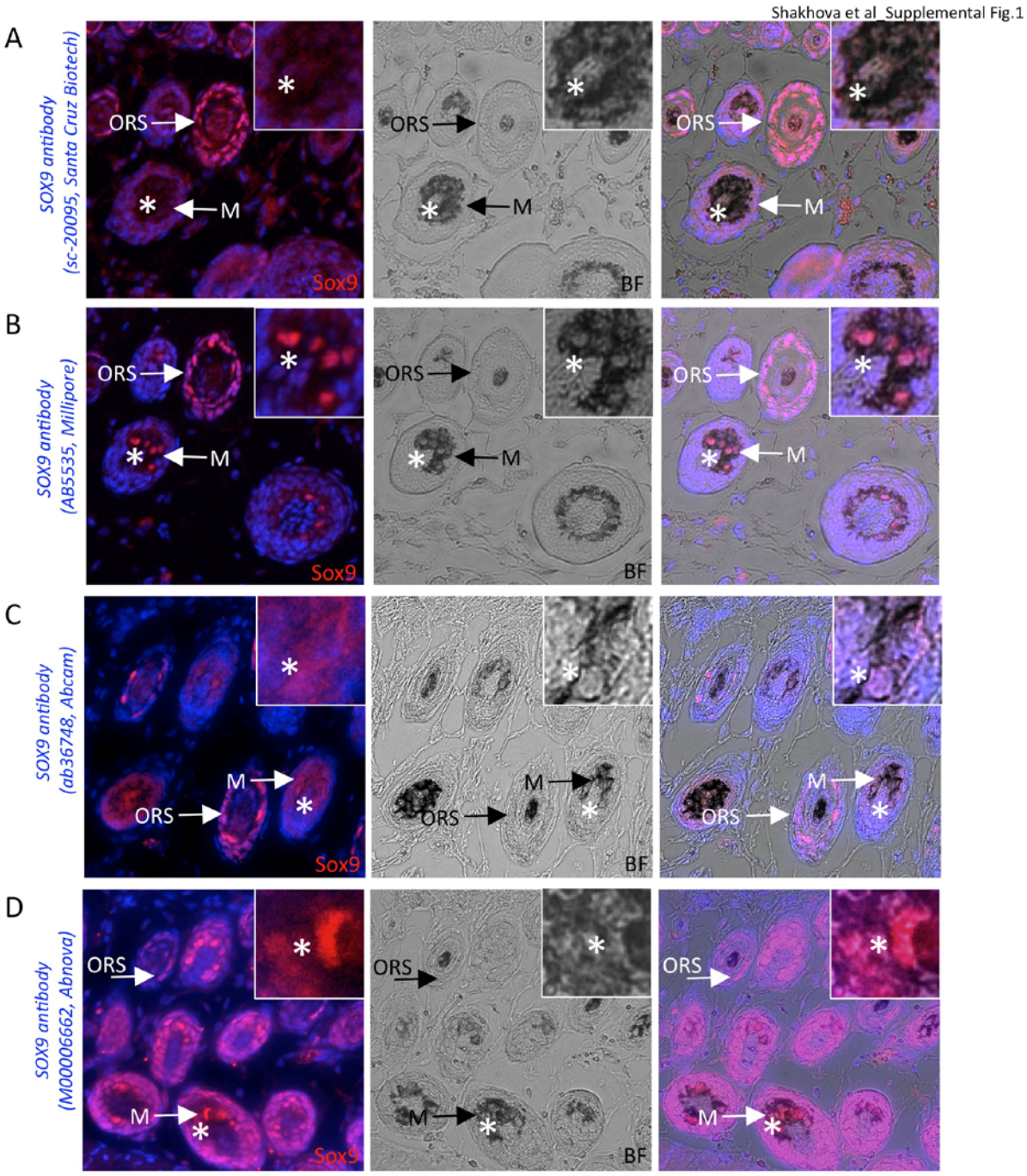
Author Contribution

Conceived and designed the experiments: OS PCheng RS GM LS. Performed the experiments: OS PCheng DZ PMishra CM TG SD PMeltzer SMS JD JH DMP PCinelli. Analyzed the data: OS PCheng JD DMP PCinelli LS. Contributedreagents/materials/analysis tools: HM MW MPL RD RS. Wrote the paper: OS LS

Supporting Information

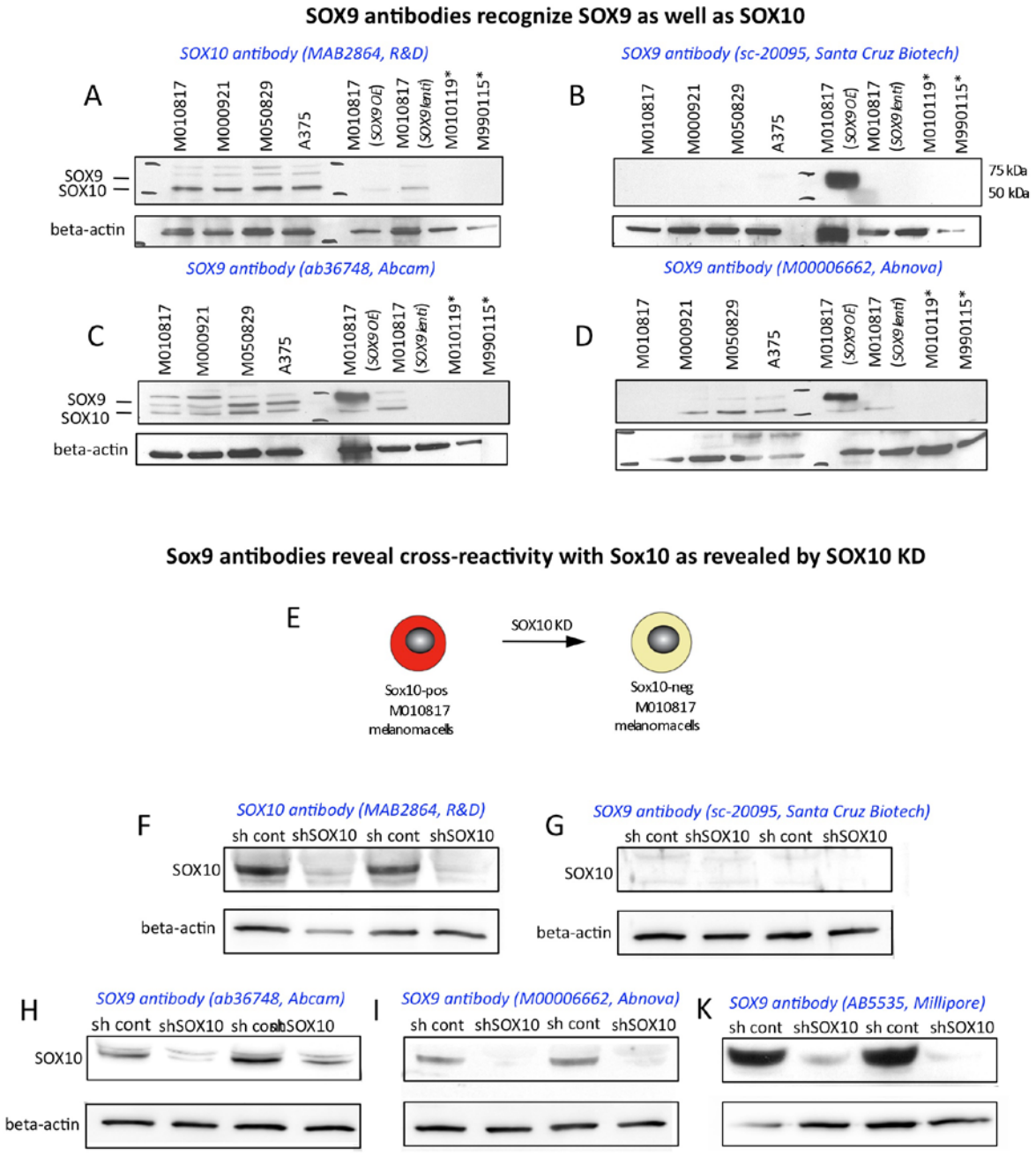


E

SOX9 Ab	Santa Cruz *sc-20095*	Millipore *AB5535*	Abcam *ab36748*	Abnova *M00006662*
ORS	pos	pos	pos	pos
Mel HF bulb	neg	pos	pos	pos

Supplementary Figure 1

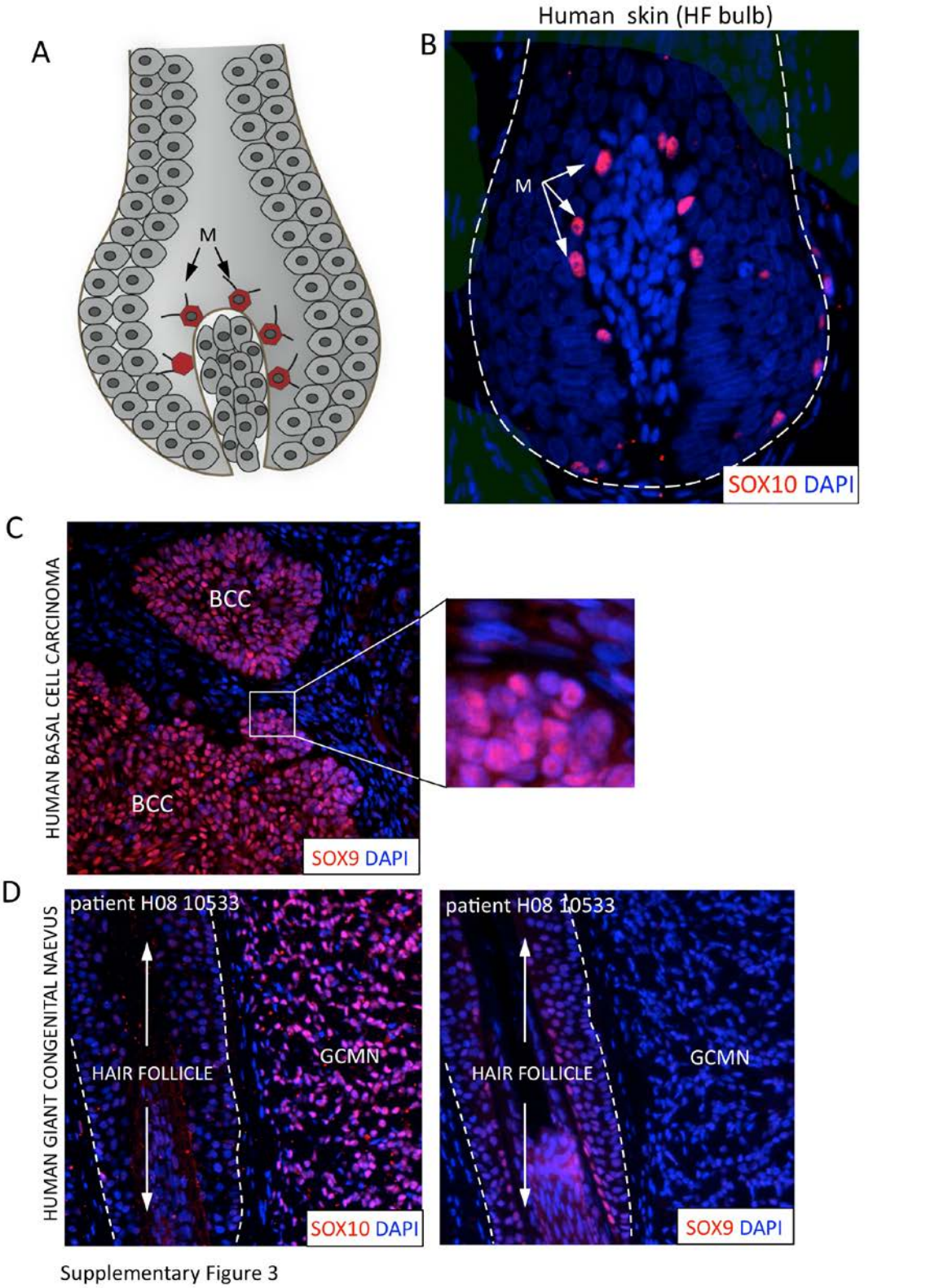
Supplementary Figure 1. Immunohistochemical analysis of the cross-reactivity of anti-Sox9 antibodies to Sox10 in the mouse skin. A-D, Immunostaining for Sox9 (red) using different anti-Sox9 antibodies, namely anti-Sox9 from Santa Cruz (A), Millipore (B), Abcam (C) and Abnova (D). E, Summary of immunohistochemical analysis using anti-Sox9 antibodies from different sources. ORS, outer rooth sheath; M, melanocytes.



Supplementary Figure 2

Supplementary Figure 2. Assessing the cross-reactivity of anti-SOX9 antibodies to SOX10. A-D, Western blots demonstrating the cross-reactivity of anti-SOX9 antibodies (Abcam, Abnova and Millipore) to SOX10 and specificity to SOX9 (A-D). **E,** A schematic illustration of the experiment used to test anti-

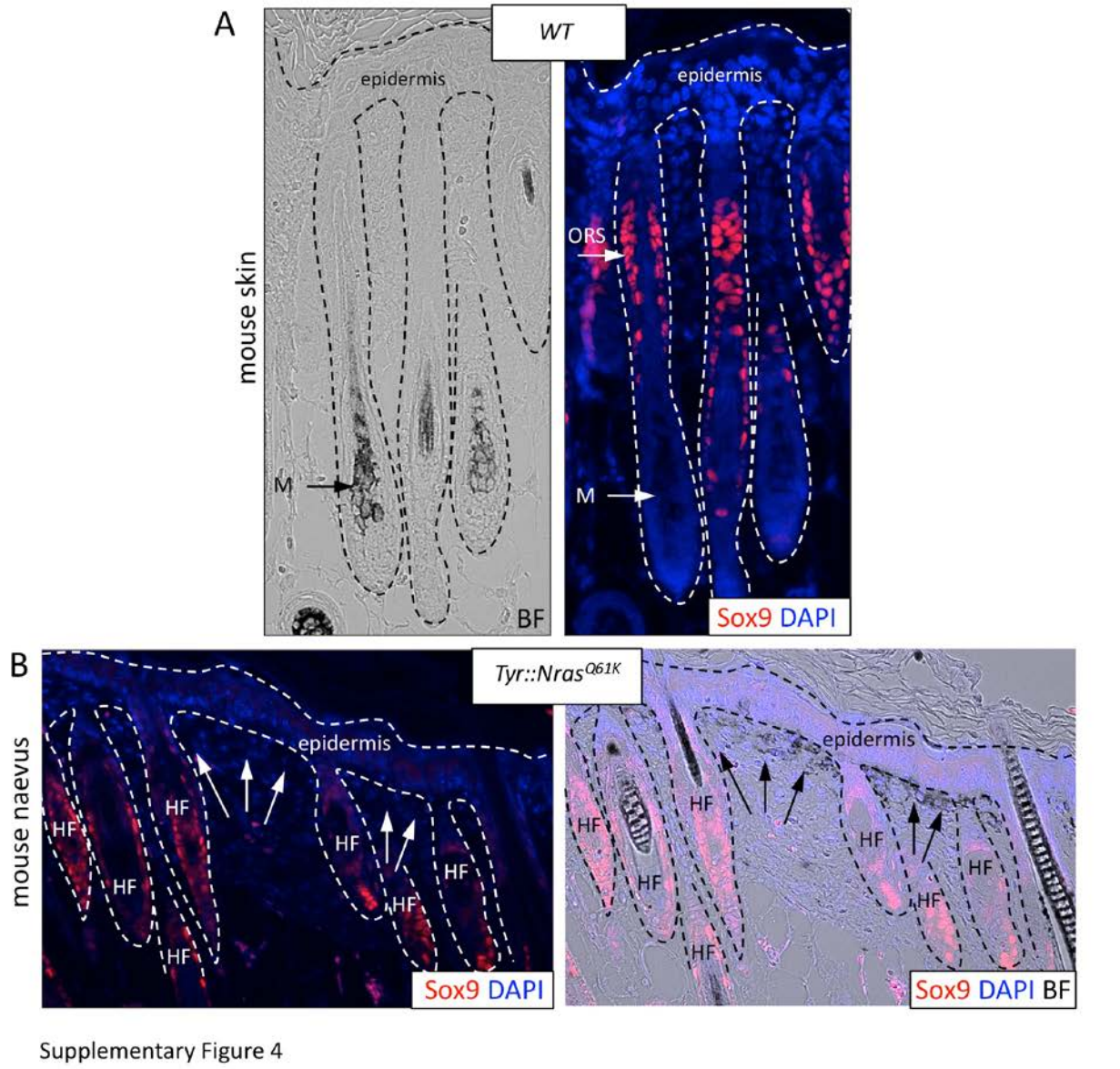
SOX9 antibodies. F–K, Western blots demonstrating the cross-reactivity of anti-SOX9 antibodies using protein homogenates isolated from melanoma cells transfected with either sh control or sh SOX10 vectors.



Supplementary Figure 3

Supplementary Figure 3. SOX10 and SOX9 expression in human melanocytic and epithelial lineages. **A,** A schematic representation of the location of melanocytes in the hair follicular bulb. **B**, SOX10 expression in the hair follicular bulb. **C,** SOX9 expression in human basal cell carcinoma. **D**, Analysis of the expression of SOX10 and SOX9 in the human giant congenital naevi (patient H08 10533). Adjacent sections were stained with anti-SOX10 and anti---SOX9 antibodies. Note the positive staining for SOX9 in the hair follicle. BCC, basal cell carcinoma; GCMN, giant congenital melanocytic naevi; M, melanocytes.

Shakhova et al_Supplemental Fig.4

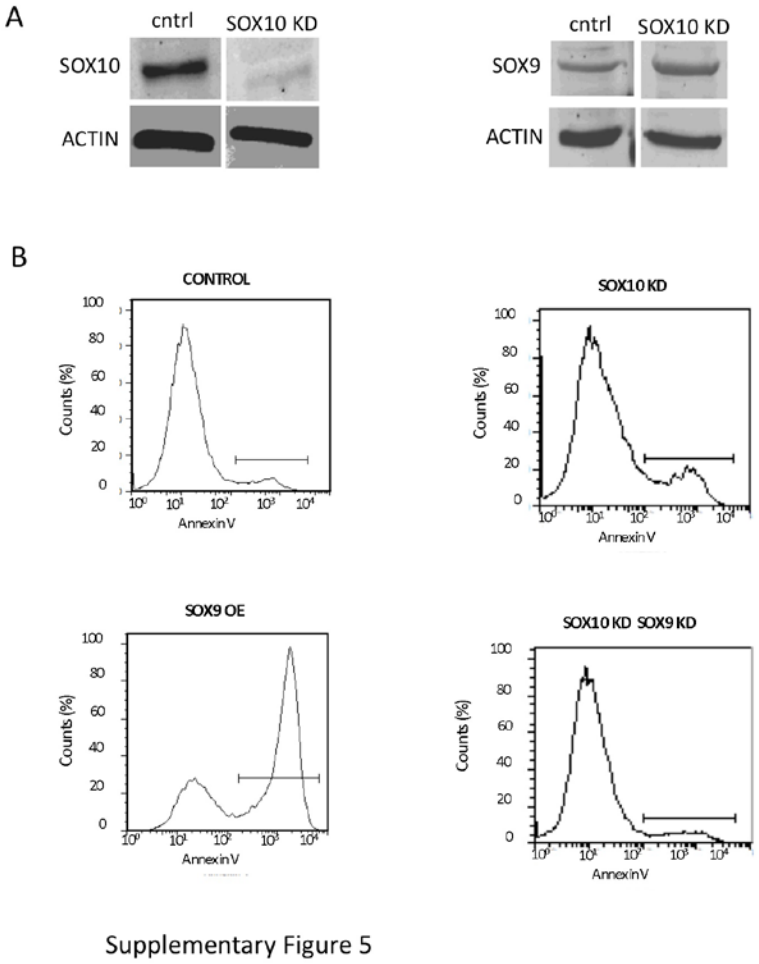


Supplementary Figure 4

Supplementary Figure 4. Sox9 is not expressed in murine melanocytes and cells of giant congenital naevi in the postnatal mouse skin. A, Bright field picture (left panel) showing the pigmented melanocytes located in the hair follicular bulb. Immunostaining for Sox9 (red) demonstrating that Sox9 is expressed in the epithelial cells of the hair follicle (outer root sheath) but not in the

pigmented melanocytes. B, Immunostaining for Sox9 (red) demonstrating the expression of Sox9 in the outer rooth sheath and the absence of Sox9 expression in the cells of giant congenital naevi in *Tyr::Nras^{Q61K}* mouse. BF, bright field; HF, hair follicle, M, melanocytes; ORS, outer root sheath.

Shakhova et al_Supplemental Fig5



Supplementary Figure 5

Supplementary Figure 5. SOX9 and SOX10 play antagonistic roles in human melanoma cells. A, Western blot analysis demonstrating that SOX9 expression is upregulated upon SOX10 knockdown in human melanoma cell lines. **B,** FACS analysis of apoptosis in M010817 melanoma cell line. M010817 control cells, M010817 SOX10 KD cells, M010817 SOX9 OE and M010817 SOX10 KD SOX9KD cells were analyzed for the number of Annexin V-positive cells. KD, knockdown; OE, overexpression.

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7. Methylation dependent SOX9 expression mediates invasion in human melanoma cells and is a negative prognostic factor in advanced melanoma

Contribution

In this publication, I performed the majority of the experiments and analysis and wrote the paper for which I was made first author.

This study was published in *Genome Research; Cheng et al. 2015.*

Abstract

Background

Melanoma is the most fatal skin cancer displaying a high degree of molecular heterogeneity. Phenotype switching is a mechanism that contributes to melanoma heterogeneity by altering transcription profiles for the transition between states of proliferation/differentiation and invasion/stemness. As phenotype switching is reversible, epigenetic mechanisms, like DNA methylation, could contribute to the changes in gene expression.

Results

Integrative analysis of methylation and gene expression datasets of five proliferative and five invasion melanoma cell cultures revealed two distinct clusters. *SOX9* was methylated and lowly expressed in the highly proliferative group. *SOX9* overexpression resulted in decreased proliferation but increased invasion *in vitro.* In a B16 mouse model, sox9 overexpression increased the number of lung metastases. Transcriptional analysis of *SOX9*-overexpressing melanoma cells revealed enrichment in epithelial to mesenchymal transition (EMT) pathways. Survival analysis of the The Cancer Genome Atlas melanoma dataset showed that metastatic patients with high expression levels of *SOX9* have significantly worse survival rates. Additional survival analysis on the targets of *SOX9* revealed that a majority of SOX9 down-regulated genes have survival benefit for metastatic patients.

Conclusions

Our genome wide DNA methylation and gene expression study of 10 early passage melanoma cell cultures revealed two phenotypically distinct groups. One of the genes regulated by DNA methylation between the two groups was *SOX9*. *SOX9* induces melanoma cell invasion and metastasis and decreases patient survival. A number of genes down regulated by *SOX9* have a negative impact on patient survival. In conclusion, *SOX9* is an important gene involved in melanoma invasion and negatively impacts melanoma patient survival.

Keywords: melanoma, DNA methylation, phenotype switching, SOX9, invasion

Introduction

Melanoma is an aggressive skin cancer that originates from melanocytes, i.e. pigment cells that reside in the basal layer of the epidermis and are derived from the neural crest during early development [1]. It is the most life-threatening neoplasm of the skin and is considered a major health problem due to rising incidence and mortality rates [2, 3]. Melanoma is a tumor with a high degree of heterogeneity and this phenotypic heterogeneity is reversible [4-7]. In addition to being a challenge for basic research, melanoma plasticity is a major hurdle for successful treatment [8]. Investigating the molecular basis of phenotypic heterogeneity is crucial to better understand melanoma progression and should provide useful insights for the development of more effective therapies.

In an effort to elucidate the molecular mechanisms of melanoma progression, significant differences have been detected between melanoma cells from the same lesion [4, 6, 9]. We and others have found that melanoma cells generally express two distinct gene expression signatures, that these signatures correlate with *in vitro* characteristics and these phenotypes are reversible depending on their cellular microenvironments [10-12]. One signature is characterized by the upregulation of several melanocytic genes like *MITF*, *TYR*, and *DCT*. These melanoma cells are highly proliferative and weakly invasive *in vitro* and so are named the proliferative phenotype. The other signature is characterized by the upregulation of many mesenchymal genes such as *WNT5A*, *TGFβ*, and *FGF2*. In contrast to the proliferative cells, these cells are highly invasive but have a low proliferative capacity *in vitro* and are thus named the invasive phenotype. Meta-analysis of all available melanoma microarray datasets available on the NCBI GEO database confirmed these two gene signatures in 86% of the 536 melanomas [13]. Immunohistochemical analyses for MITF and WNT5A, markers of the proliferative and invasive phenotype respectively, of human primary and metastatic melanomas displayed an anti-correlative staining pattern confirming that these phenotypes exist *in vivo* [14]. Together these findings culminated in the phenotype switching model for melanoma progression, in which melanoma cells respond to changing microenvironmental signals, such as hypoxia, by reprogramming their gene expression patterns to switch between states of proliferation and invasion [15] [9]. Thus, phenotype switching has important implications in melanoma progression. Invasive phenotype cells characterized by low MITF expression, have stem-like properties [16], including the ability to initiate tumors with high efficiency [17]. Consequently, tumors comprise a mix of MITF-positive and negative melanoma cells [18].

DNA methylation provides a stable and heritable gene regulatory mechanism for which melanoma cells could alter the expression of many genes [19]. Aberrant DNA methylation is a mechanism known to cause tumorigenesis [20]. Tumor suppressor genes become silenced by hypermethylation of their promoter region, thus promoting tumorigenesis. Global hypomethylation has been observed in many cancers including melanoma to decrease with progression of the disease [21-23]. DNMT3a and DNMT3b, the *de novo* DNA methyltransferases, were shown to have increased expression in metastatic melanomas compared to primary melanomas [24]. Another group showed that DNMT3a is required for melanoma development and metastasis in a melanoma mouse model [25]. Several signaling pathways have been shown to be deregulated as a result of aberrant DNMT-dependent methylation in melanoma, which include MAPK, WNT, PI3K, pRB, and pathways in cell cycle, apoptosis, invasion and metastasis [26]. Progressive global DNA hypomethylation has been observed in malignant melanocyte transformation, and surprisingly transformation was blocked in the presence of 5-Aza-2-deoxycytidine (Decitabine, Aza), a DNMT inhibitor [22]. It would suggest that targeted hypomethylation is required for malignant transformation and not overall global hypomethylation caused by Aza treatment. This is supported by our observation that treating proliferative melanoma cells with Aza had no measureable effect on their invasive abilities (data not shown). 5-Aza-2-deoxycytidine treatment of various melanoma cell lines was shown to increase SOX9 expression and induce expression of p27 and p21 [27]. SOX9 is a transcription factor involved in neural crest specification [28] and SOX9 overexpression in melanoma cell lines have been shown to induce cell cycle arrest in a p21 dependent manner [29]. Taken together, it would suggest that DNA methylation has a crucial role in malignant transformation and progression by altering the landscape of the methylome to promote tumor progression, and SOX9 is one of the targets of DNA methylation that induces cell cycle arrest. In this study, we examine the expression of the DNMTs between the proliferative and invasive melanoma cell cultures and describe the differential melanoma methylome by MeDIP-chip. We confirm that SOX9 expression is regulated by DNA methylation and has a role in cell cycle regulation, invasion *in vitro* and *in vivo* and could be a prognostic marker for overall survival in metastatic melanoma patients.

Results

Proliferative melanoma cells have higher levels of global DNA-methylation

We have previously established melanocytic markers like MLANA to distinguish between the proliferative and invasive phenotype on a cohort of primary melanoma cell cultures [11, 30] (Supplemental Figure 1). To investigate if methylation differences exist between the proliferative and invasive melanoma phenotypes, we compared five proliferative (i.e. M000921, M010817, M080423, M980513, M050829) and five invasive (i.e. M990115, M010119, M080201, M080307, M080310) melanoma cell cultures for expression of de novo DNA methyltransferases DNMT1, 3a, and 3b (Figure 1A). We observed that the invasive phenotype melanoma cell cultures had about 51.1% less expression of DNMT1 as compared to the proliferative phenotype melanoma cell cultures (Figure 1A). DNMT3b had about 50% less protein expression in the proliferative melanoma cell cultures compared to the invasive melanoma cell cultures (Figure 1A). However, DNMT3a was not differentially expressed between the proliferative and invasive phenotype (Figure 1A). Global methylation analysis by methyl-cytosine ELISA showed that the invasive phenotype cells have significantly less DNA methylation in their genome compared to the proliferative phenotype cells, 13.0% to 20.9% respectively (Figure 1B). This raises the possibility that differential methylation exists between the proliferative and invasive phenotype.

A 73-gene signature is significantly differentially methylated and expressed in proliferative melanoma cells

The difference in global methylation levels and protein expression of DNMT1 and DNMT3b prompted us to investigate the methylation profiles of the proliferative and invasive phenotype melanoma cells. To determine which CpG islands were differentially methylated between the proliferative and invasive phenotypes, we immunoprecipitated methylated DNA from five proliferative and five invasive melanoma cell cultures by MeDIP [31] followed by hybridization to Nimblegen Human DNA Methylation 3x720K CpG Island Plus RefSeq Promoter Arrays. This array contains 720,000 probes for 22,532 promoter regions and 27,728 CpG islands. We calculated the differential methylation levels between the five proliferative and five invasive melanoma cells with a sliding window ANOVA test with the R package DMR supplied from Nimblegen. We found 406 gene promoters to be significantly and differentially methylated between the proliferative and invasive phenotypes (Figure 1C). 320 promoter regions were hypermethylated in the proliferative phenotype and 86 promoter regions were hypermethylated in the invasive phenotype. The greater number of hypermethylated regions in the proliferative phenotype would be consistent with the global methylation data.

Gene expression data for the 10 melanoma cell cultures previously generated by us [13] were reanalyzed for differential gene expression between the proliferative and invasive phenotypes using the R package limma [32]. A total of 1750 genes were differentially expressed between the proliferative and invasive phenotype (Fold Change > 2, FDR corrected $p < 0.05$) (Figure 1D). We then analyzed the relationship between the promoter methylation status and mRNA expression levels for all genes in both datasets. Genes were filtered for a peak score > 2 for methylation, fold change > 2 for gene expression and an FDR-corrected p-value < 0.05. A total of 73 genes showed both significant differential DNA methylation and significant differential expression between the proliferative and invasive phenotype (Supplemental Table 1). 62 genes from the proliferative phenotype had hypermethylated promoters and low RNA expression and 11 genes in the invasive phenotype had hypermethylated promoters and low RNA expression as compared to the proliferative phenotype. This suggests that methylation has a role in regulating a portion of the genes differentially expressed between the proliferative and invasive phenotype. We hypothesized that the 73 genes with both differential DNA methylation and mRNA expression between the proliferative and invasive melanoma cells were likely to be true targets of epigenetic regulation in melanoma. To determine which groups of genes were functionally important, we performed pathway analysis of the 73 genes on MetaCore. We looked for enrichment of pathways under GO processes, process networks and Pathway maps (Supplemental Table 2). Interestingly, we observed significant enrichment in pathways involved in EMT, melanoma and cell differentiation. We decided to focus on *SOX9* for validation due to its known function in melanocyte differentiation and melanoma progression [29, 33, 34].

Figure 1

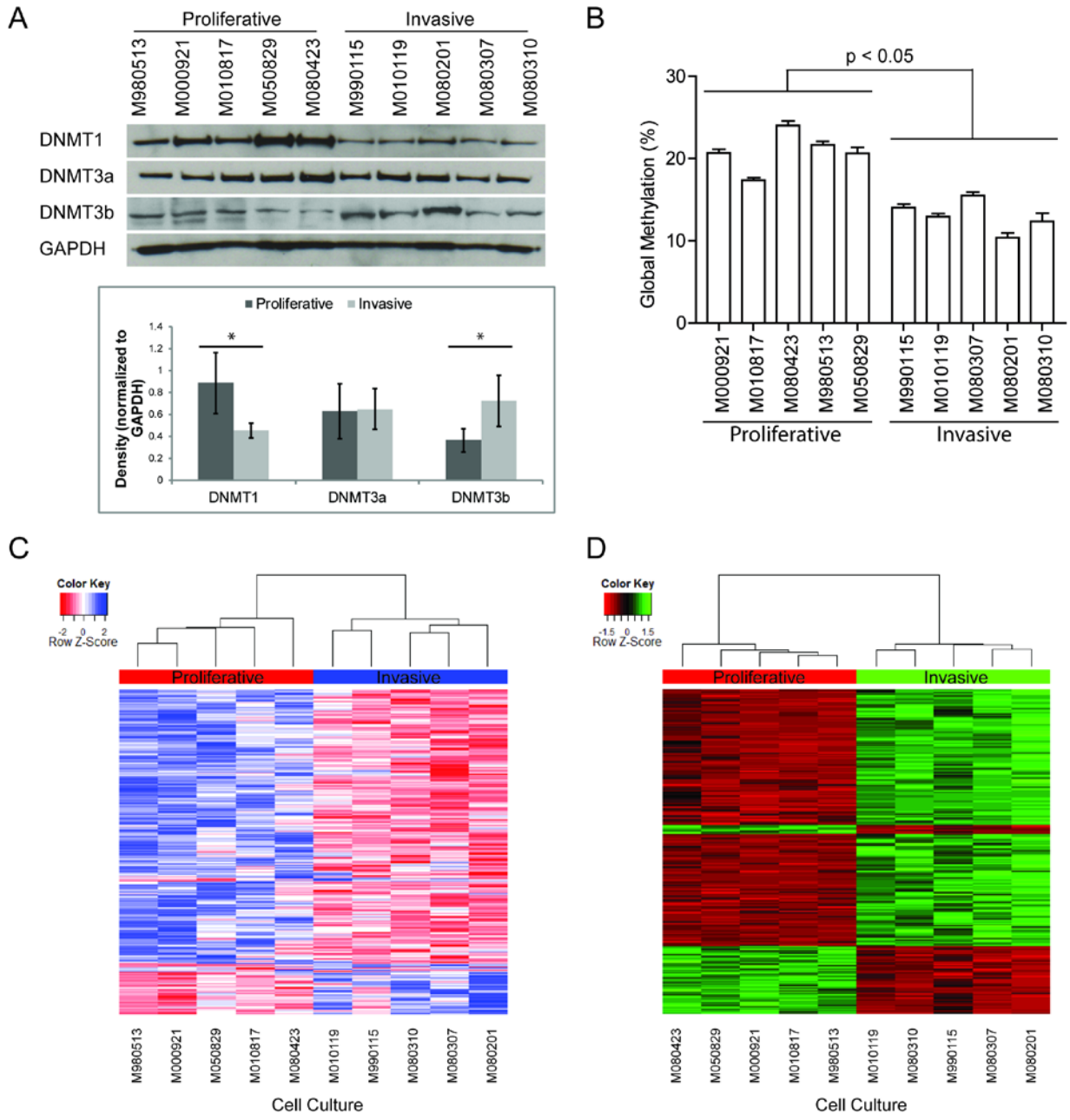

Figure 1. Phenotype specific expression of DNMTs and DNA methylation patterns. (a) Western blot for DNMT1 and DNMT3a and DNMT3b on five proliferative phenotype and five invasive phenotype cell lysates. GAPDH was used as a loading control. Optical density of each band was measured and normalized to GAPDH intensity. (b) Global methylation analysis by ELISA for methyl-cytosine on five proliferative cell cultures and five invasive cell cultures. There was a significant difference ($p < 0.05$, Student's t-test) in global methylation between the proliferative and invasive phenotype melanoma cells. (c) Heat map representing the 406 gene promoters differentially methylated between the proliferative and invasive phenotype cultures. (d) Heat map representing the top 250 genes differentially expressed between the proliferative and invasive phenotype.

Sox9 expression is silenced in proliferative melanoma cells through promoter DNA-methylation

From the methylation array, the area that had the most enrichment for methylation was about 2kb upstream of the *SOX9* promoter, thus we validated the CpG island located there via sequencing of bisulfite-treated genomic DNA in the 10 melanoma cell cultures (Figure 2A). There are three predicted transcription factor binding sites in that upstream promoter region of *SOX9* for *MEF2*, *E2F* and *HNF3B*. We analyzed the DNA methylation status of a cluster of 17 CpGs across a 283-bp region and 15 CpGs across a 256-bp region of a CpG island, located approximately −2500 bp to −2000 bp upstream of the *SOX9* transcriptional start site. The majority of CpGs in both regions of the *SOX9* promoter were consistently methylated in the proliferative phenotype melanoma cell cultures and consistently unmethylated in the invasive phenotype melanoma cell cultures.

To confirm that promoter DNA hypermethylation correlated with transcriptional silencing of SOX9, we assessed mRNA levels using real-time RT-PCR in the ten melanoma cell cultures. *SOX9* mRNA was expressed robustly in the invasive phenotype melanoma cell cultures compared to the proliferative phenotype melanoma cell cultures ($p < 0.05$) (Figure 2B). Protein expression of *SOX9* was detected in all invasive phenotype melanoma cell lysates, but little to no expression of *SOX9* was seen in the proliferative phenotype melanoma cell lysates (Figure 2C). To validate that *SOX9* is indeed regulated by DNA methylation, we treated the five proliferative phenotype melanoma cell cultures with 5 µM 5-Aza-2'-deoxycytidine (a DNMT inhibitor) for 72 hours. Reexpression of *SOX9* was detected by western blot (Figure 2D). Thus, *SOX9* expression is regulated by DNA methylation between the proliferative and invasive phenotype.

Figure 2

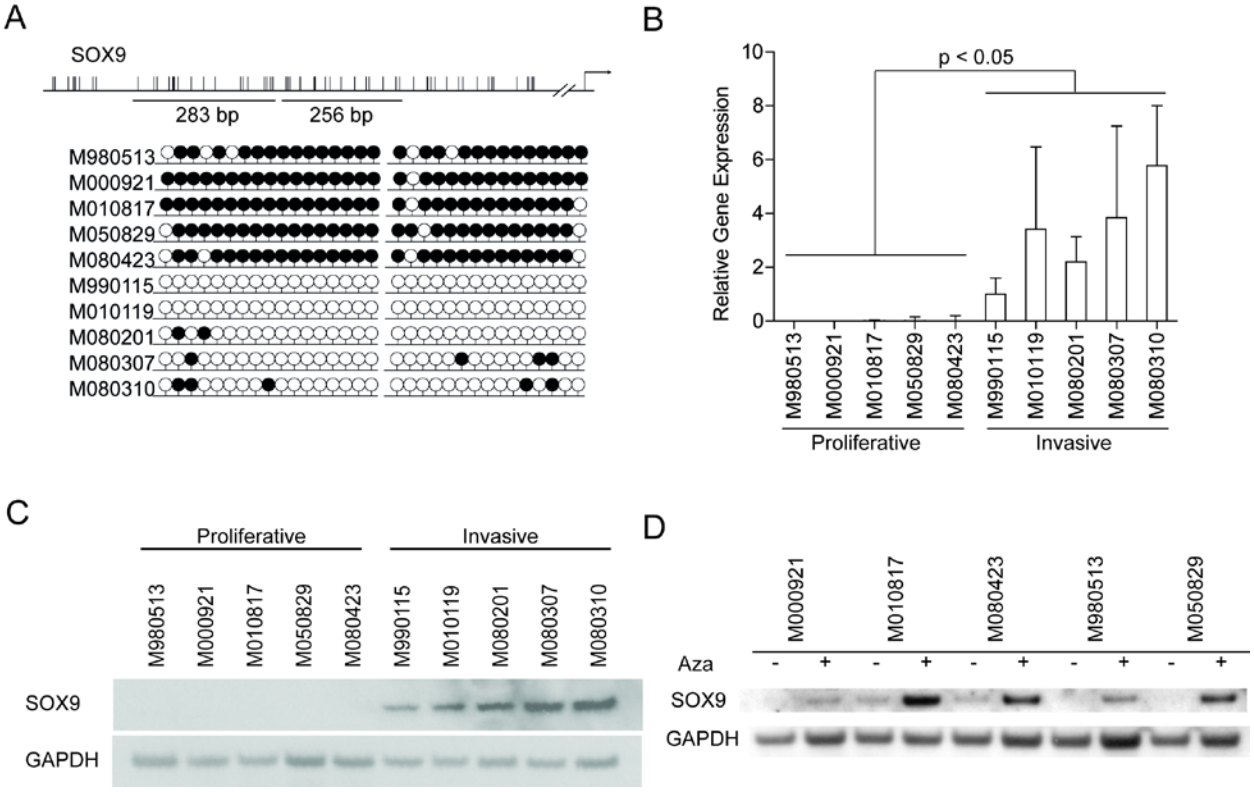

Figure 2. Validation of SOX9 methylation. (a) Lollipop diagrams of bisulphite sequencing of SOX9 promoter. Black lollipops are methylated CpGs; white lollipops are unmethylated CpGs. A minimum of 5 clones were sequenced for each cell culture. (b) mRNA expression of SOX9 normalized to the housekeeping gene RPL28 across ten melanoma cell cultures. Results are presented as mean +/- s.d., n=3. Statistical significance of differential expression between the proliferative and invasive phenotype cell cultures was determined by Student's t-test. (c) Western blot for SOX9 in ten melanoma cell cultures. GAPDH served as loading control. (d) Western blot for SOX9 of five proliferative melanoma cell cultures treated with 5-aza-2-deoxycytidine (Aza)

SOX9 mediates proliferation and invasion in melanoma cell cultures

SOX9 is expressed in the invasive phenotype and we have previously described the greater invasive potential of invasive versus proliferative phenotype melanoma cells [30, 35]. We hypothesized that some of the differentially expressed genes could have a role in generating this invasive capacity, thus we wanted to see if *SOX9* would have a role in invasion. SOX9 was knocked down with siRNA, and then the invasive ability of two invasive phenotype melanoma cell cultures was measured: M080201 and M080310. Treatment with two independent siRNAs for *SOX9* achieved about 70% knockdown of *SOX9* mRNA in M080201 and M080310 (Figure 3A). The invasive capacity of M080201 and M080310 decreased significantly ($p < 0.05$) from 30% to 11.5% and from 64.8% to 36.6% respectively after 48 hours treatment with siRNA

targeting *SOX9* (Figure 3B, C). Proliferation was unaltered from *SOX9* knockdown (data not shown).

Consistent with this observation, we overexpressed *SOX9* in proliferative phenotype melanoma cell cultures (i.e. M010817 and M980513) by lentiviral transfection (Figure 3D). Overexpression of *SOX9* was previously shown to drive melanoma cells into cell cycle arrest [29]. We measured proliferation and cell cycle progression by EdU and PI staining respectively and observed the cells transfected with vector have 50.5% in G1 phase, 37% in S phase, and 12.5% in G2/M phase, whereas the cells overexpressing SOX9 have 64.8% in G1 phase, 17.7% in S phase, and 17.5% in G2/M phase ($p<0.05$) (Figure 3E, F). The invasive capacity of M010817 and M980513 were significantly increased from 0.95% to 9.0% ($p<0.05$) and from 0.67% to 4.8% ($p<0.05$) from *SOX9* overexpression (Figure 3G, H). In concordance with previously published data on *SOX9* in melanoma, we also see G1/G0 arrest when SOX9 is overexpressed along with increased invasion.

Figure 3

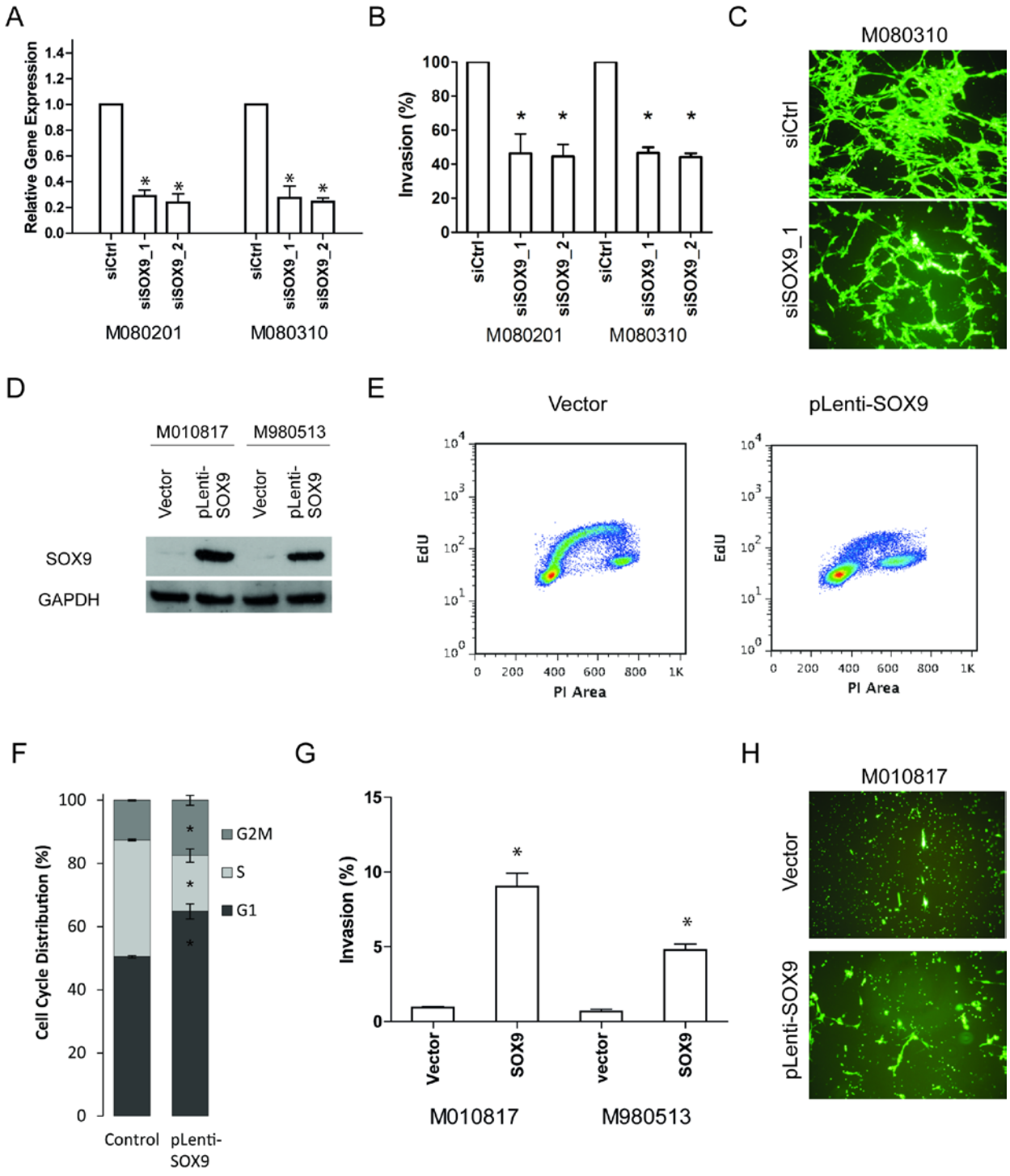

Figure 3. SOX9 mediates invasion and cell cycle arrest. (a) SOX9 knockdown by siRNA in two melanoma cell cultures M080201 and M080310. (b) Boyden chamber assay for siSOX9 knockdown in M080201 and M080310. (c) Representative picture of the Boyden chamber assay for knockdown of SOX9 in M080310. Top panel shows M080310 cells treated with control siRNA (siCtrl). Bottom panel

shows M080310 cells treated with siSOX9_1. (d) SOX9 overexpression by lentiviral expression in proliferative phenotype melanoma cell cultures M010817 and M980513. (e) Proliferation assay by EdU pulse in cell cultures transfected with empty vector and pLenti-SOX9. (f) Cell cycle analysis by PI staining for cell cultures transfected with empty vector and pLenti-SOX9 (top and bottom left panels). Quantitation of cell cycle analysis (* = $p < 0.05$, right panel). (g) Boyden chamber assay for SOX9 overexpressing melanoma cell cultures M010817 and M980513 (* = $p < 0.05$). (h) Representative picture of the Boyden chamber assay for SOX9 overexpression in M010817. Top panel shows M010817 transfected with empty vector. Bottom panel shows M010817 transfected with SOX9.

SOX9 induces a partial invasive phenotype in proliferative melanoma cells

To determine the effect of *SOX9* overexpression on the proliferative phenotype, we performed microarray analysis of M010817 cells overexpressing *SOX9*. We detected 643 genes down regulated at least 2 fold and 450 genes up regulated at least 2 fold ($p < 0.05$ (Figure 4A, Supplemental Table 3). We overlapped the gene signature from the SOX9 overexpression microarray with the gene signature from the 10 melanoma cell culture microarray to ask if *SOX9* induced genes are enriched in the invasive phenotype. There were 98 genes that were up-regulated and 55 genes that were down-regulated in both the *SOX9* overexpression and invasive phenotype gene sets. Hypergeometric distribution of the overlap of the *SOX9* microarray with 10 melanoma cell culture array was significant ($p < 0.001$) (Figure 4B). Thus, *SOX9* apparently regulates about 10% of the genes that define the invasive phenotype gene set. This suggests that *SOX9* activation contributes to the invasive phenotype but other factors are also required for the full transition.

Figure 4

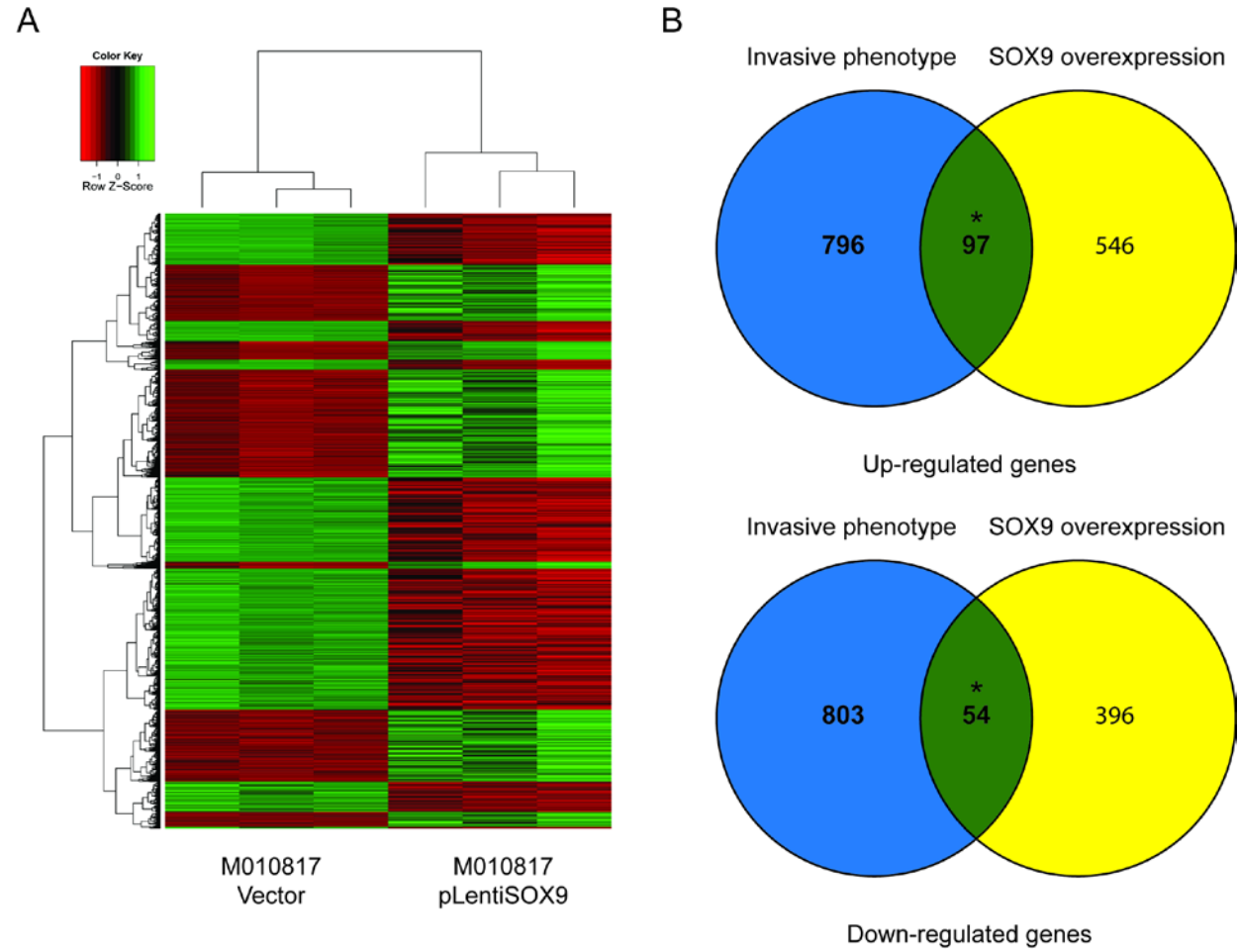

Figure 4. SOX9 microarray (a) Heat map of M010817 cells overexpressing SOX9. (b) Overlap of up-regulated genes and down-regulated genes between the SOX9 microarray and 10 melanoma cell culture microarray.

In vivo function of SOX9 overexpression

To examine the effects of SOX9 *in vivo,* we utilized the B16F1 mouse melanoma cells which do not express sox9 and are known not to metastasis in a tail vein injection assay. We transfected murine sox9 transiently into the B16F1 cells and monitored its expression over 288 hours. Expression of sox9 decreases over time but protein is still detectable at 288 hours (Figure 5A). To assess the *in vivo* metastatic potential of sox9, C57BL/6J mice were intravenously injected with B16F1 cells transfected with sox9 and empty vector (Figure 5B). 12 days after injection, the mice were sacrificed and the lungs were analyzed for tumor nodules. B16F1 cells expressing sox9 had significantly more metastases compared to control, $p < 0.05$.

Figure 5

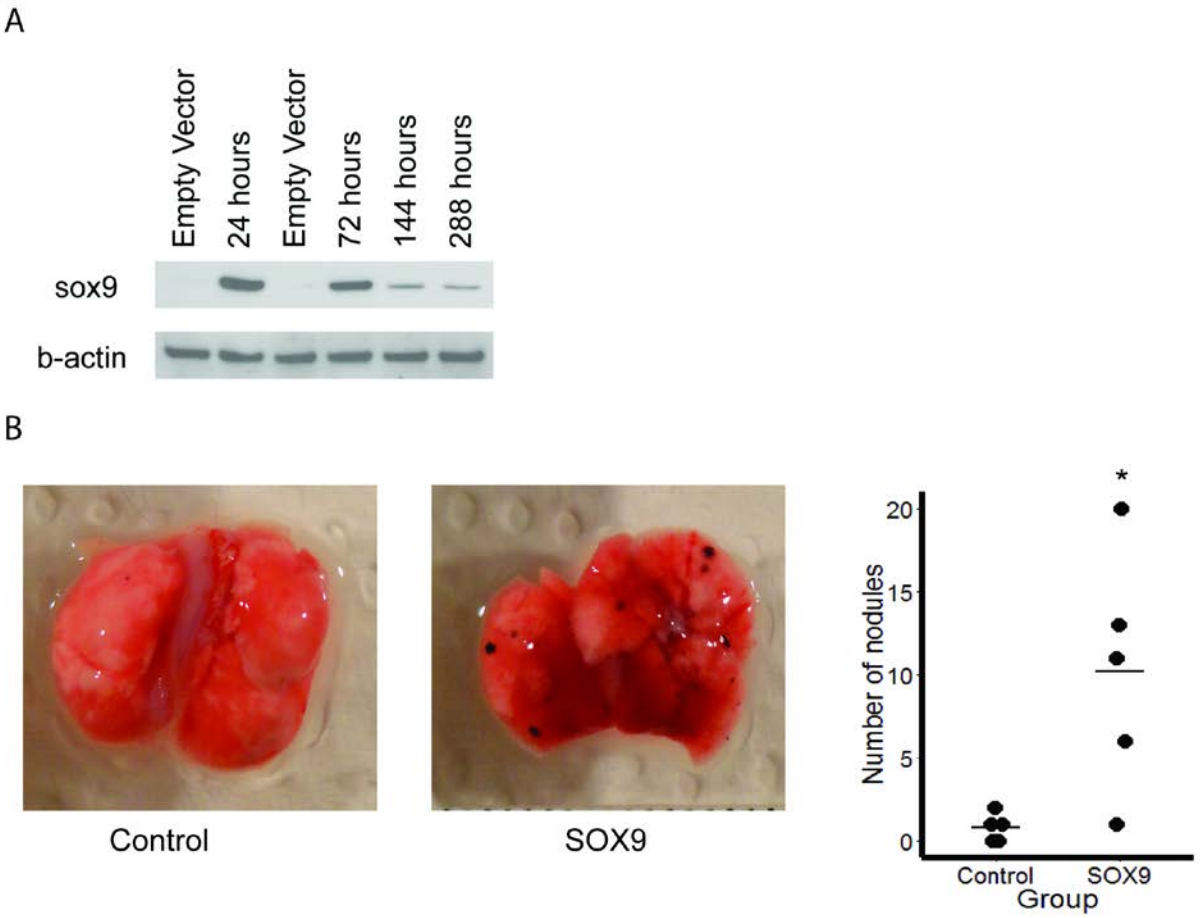

Figure 5. *In vivo* function of sox9 overexpression. (a) Western blot analysis of transient sox9 overexpression in B16F1 up to 12 days. (b) Representative pictures of B16F1 cells intravenously injected into C57BL/6J mice 12 days after injection (n = 5 mice per group). Quantification (right graph). * = $p < 0.05$ Mann-Whitney U test.

Validation with TCGA melanoma dataset

The Cancer Genome Atlas (TCGA) has a melanoma dataset available for public access which contains over 300 tissue samples with RNAseq, DNA methylation and clinical data. To validate our claim that *SOX9* is regulated by DNA methylation, we performed a correlation analysis of *SOX9* expression to *SOX9* promoter methylation using the data from the TCGA database. Three consecutive probes cg10471574, cg21049501, and cg06234051 in the *SOX9* promoter region have an anti-correlative association with the expression of *SOX9*, r= -0.58, -0.61, and -0.71 respectively (Figure 6A). Since high DNA methylation of *SOX9* is correlated with low *SOX9* expression it provides strong evidence that DNA methylation regulates *SOX9* expression *in vivo*.

The melanoma dataset contains 68 primary samples and 268 metastatic samples. The 268 metastatic samples are comprised with 172 lymph node tumors, 59 regional cutaneous or subcutaneous metastases and 37 distant metastases. Due to the diversity of this dataset we analyzed the primary and metastatic samples individually. We segregated the population into thirds by *SOX9* expression. We compared the upper and lower thirds for our analysis labelling them *SOX9* high and *SOX9* low. In both primary and metastatic datasets, the *SOX9* high group had at least 3 times more expression than the *SOX9* low group. We interrogated clinically relevant factors such as TNM staging, age, gender, and tumor type between the *SOX9* high and *SOX9* low group in the primary and metastatic datasets (Table 1, Supplemental Table 4). All parameters were statistically insignificant as tested by the Chi-squared test and t-test for age in the primary melanoma dataset. Only two clinical parameters were significant in the metastatic dataset. T1 was significant 2 vs 11 ($p = 0.013$) in the *SOX9* low versus *SOX9* high respectively. N0 was also significant 26 vs 44 ($p = 0.031$) in the *SOX9* low versus *SOX9* high respectively.

Table 1 – Clinical charactistics of TCGA melanoma SOX9 population

Clinical Parameter	SOX9 low	SOX9 high	p value	
T0	15	13	0.706	
T1	2	11	0.013	*
T2	17	17	1	
T3	20	13	0.223	
T4	16	16	1	
N0	26	44	0.031	*
N1	16	9	0.162	
N2	15	9	0.221	
N3	12	9	0.513	
NX	1	0	1	
M0	67	64	0.793	
M1	2	6	0.157	
Female	29	25	0.586	
Male	45	49	0.68	
Lymph Node	48	49	0.919	
Regional Cutaneous or Subcutaneous Metastasis	17	14	0.59	

Distant Metastasis	9	11	0.655	
Age	57	59.9	0.213a	
a: Student's t-test				

Figure 6

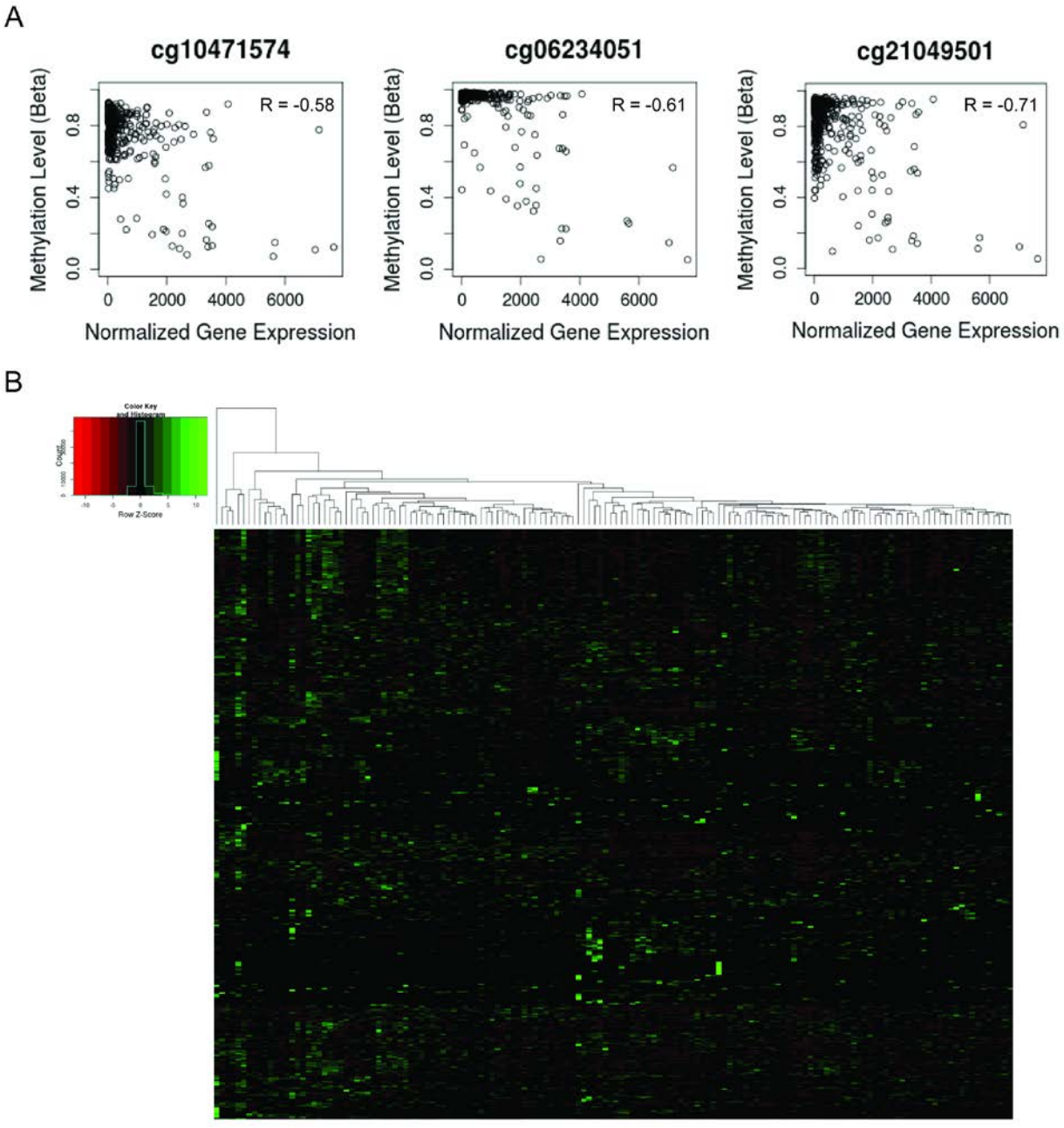

Figure 6. Validation of SOX9 methylation in TCGA and correlation with clinical features. (a) Correlation plots of RNAseq reads to B-values of methylation for SOX9. Three methylation probes of SOX9 are shown here to have significant anti-correlation of gene expression and DNA methylation. (b) Heatmap of the 427 genes differentially expressed between the SOX9 high and SOX9 low metastatic melanoma samples.

We next tested if the genes differentially expressed between the *SOX9* high and *SOX9* low patients in the primary and metastatic datasets were the same in our *SOX9* overexpression microarray. A total of 21 genes were differentially expressed between the *SOX9* high and *SOX9* low groups in the primary dataset with a minimum fold change of 2 and FDR corrected p-value < 0.05 (Supplemental Table 5). No genes from this set overlapped with the SOX9 microarray. A total of 427 genes were differentially expressed between the *SOX9* high and *SOX9* low groups in the metastatic dataset with a minimum fold change of 2 and FDR corrected p-value < 0.05 (Figure 6B, Supplemental Table 6). A total of 31 genes overlapped with the SOX9 microarray. Although the overlap was small, hypergeometric distribution of this overlap was significant ($p < 0.05$). To examine the pathways in which, *SOX9* might play a role *in vivo*, we performed pathway analysis on the 427 genes. We saw significant enrichment of many EMT pathway processes such as “Regulation of epithelial-to-mesenchymal transition (EMT)”, “TGF-beta dependent induction of EMT via SMADs”, and “Melanocyte development and pigmentation” (Figure 7A).

We interrogated if *SOX9* has a role in overall patient survival in all patients regardless of primary or metastatic disease. There was a significant difference in 10 year survival rates between the *SOX9* high and *SOX9* low groups. *SOX9* high patients had a median survival rate of 3.9 years whereas the *SOX9* low patients had a median survival time of 5.8 years ($p < 0.05$) (Figure 7B). Cox multivariate analysis was carried out to identify if age, gender, TNM stage, and tumor type were significant contributing factors for 10 year survival of *SOX9* high and *SOX9* low patients. *SOX9* expression (Hazard ratio 2.343; 95% Confidence interval 1.402 – 3.915; $p = 0.001$; SOX9 high vs SOX9 low) and T4 stage (Hazard ratio 2.145; 95% Confidence interval 1.01-4.557; $p = 0.047$; T4 vs T0) were significant (Table 2).We also segregated the patients into primary, lymph node metastasis and regional/distant metastasis and reassessed survival based on SOX9 expression. We saw that SOX9 expression in primary tumors had no effect on patient survival. However, the cohort of patients with high SOX9 expression in lymph nodes had significantly lower survival ($p = 0.03$), as in the regional/distant metastasis cohort ($p = 0.01$) (Figure 7C).

Table 2 – Multivariate Cox Regression

Covariate	HR	Lower 95%	Upper 95%	p-value	
SOX9 (SOX9 low = 0, SOX9 high =1)	2.343	1.402	3.915	0.001	***
Age	0.995	0.977	1.103	0.59	
Gender (female = 0, male =1)	0.756	0.457	1.25	0.276	
T0 (used as reference)					
T1	0.431	0.117	1.598	0.208	
T2	0.943	0.416	2.138	0.888	
T3	1.022	0.489	2.132	0.955	
T4	2.145	1.01	4.557	0.047	*
N0 (used as reference)					
N1	1.673	0.813	3.446	0.162	
N2	1.365	0.673	2.771	0.388	
N3	1.322	0.574	3.044	0.512	
M0 (used as reference)					
M1	2.023	0.672	6.09	0.21	
Lymph Node (used as reference)					
Regional Cutaneous or Subcutaneous Metastasis	0.844	0.452	1.577	0.595	
Distant Metastasis	1.83	0.957	3.499	0.068	

We tested all 31 overlapping genes for survival benefit in the metastatic dataset (Supplemental Table 7). 12 of the genes of this set were up-regulated and 19 were down-regulated when SOX9 expression was high. Most of the genes, 92% (11/12), that were up-regulated when SOX9 expression is high had no influence on patient survival, only 1 gene 7% (1/12) was associated with improved survival (Figure 7D). This suggests that the genes up-regulated by SOX9 are not direct factors for patient survival. Interestingly, 58% (11/19) of the genes down-regulated when SOX9 expression is high were associated with improved survival and the other 42% (8/19) had no influence on patient survival (Figure 7D). This suggests that SOX9 represses a group of genes important for patient survival. Taken together, high SOX9 expression leads to poor survival possibly due to the down-regulation of several genes that influence patient survival.

SOX9 binds to the promoter regions of its target genes

Using the SOX9 binding motif to screen for potential binding sites on the promoter regions from the 31 overlapping targets of the SOX9 microarray and the TCGA melanoma dataset, we found 19 of the 31 genes had a potential SOX9 binding site within a 3kb region upstream from the TSS. To determine whether SOX9 directly binds to the promoter regions of these genes, we performed ChIP analysis using SOX9 antibodies on M010817-SOX9 cells and measured SOX9 occupancy at promoter regions of TMEM158, TBX3 and FYB, for which we could design specific primers for qPCR (Figure 7E). The specificity of this assay was demonstrated by the enrichment of three known SOX9 target sequences, COL2A1 intron 1 [36], p21 [29] and SOX10 (Shahkova et al. submitted), as compared to a non-target gene (IP10). We observed a specific association of SOX9 with TMEM158, TBX3 and FYB, suggesting that TMEM158, TBX3 and FYB are direct targets of SOX9 in melanoma.

Figure 7

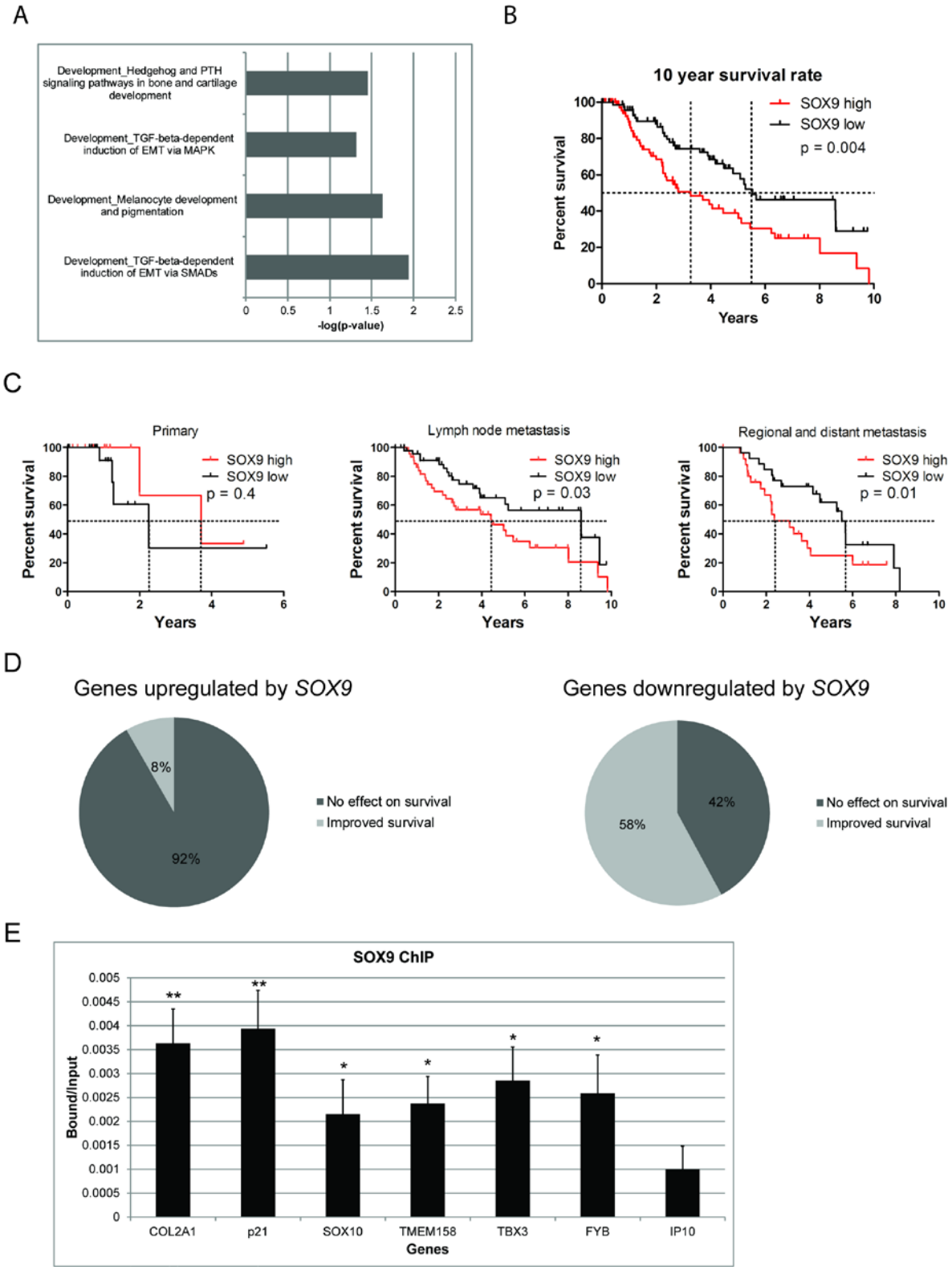

Figure 7. TCGA analysis of SOX9. (a) Pathway analysis from MetaCore for SOX9 reveals many pathways involved in EMT. (b) Median survival time for patients with high SOX9 expression is 3.9 years

(n=74). Median survival time for patients with low SOX9 expression is 5.8 years (n=74). The difference in survival is significant ($p < 0.05$). (c) Survival analysis for high and low SOX9 expression segregated into primary, lymph node and metastatic cohorts. (d) Pie charts displaying the number of SOX9 target genes that have a contribution to patient survival. (e) ChIP analysis for SOX9 binding targets. Enrichment of the promoter regions for TMEM158, TBX3, and FYB were similar to positive controls COL2A1, p21 and SOX10 and greater than negative control IP10. Data are shown as bound vs input. Error bars indicate standard error of the mean of 3 independent experiments. IgG controls not shown on graph because they were below detection limit.

Conclusion

Phenotypic, genetic, and epigenetic heterogeneity is a common feature in human melanomas [4, 5, 14, 37]. Tumor subpopulations can be transient and have been seen to switch between phenotypic states *in vivo* [4, 6, 9, 12, 38]. We have previously described two subpopulations in melanoma, the proliferative phenotype and the invasive phenotype, which are defined by specific gene signatures, *in vitro* characteristics and response to drug treatment [11, 13, 30, 35, 39]. Briefly, proliferative phenotype melanoma cells are distinguished by a high proliferative capacity and low invasive capacity and the invasive phenotype melanoma cells are distinguished by a low proliferative capacity and high invasive capacity. In this study, we found specific DNA methylation signatures for the proliferative and invasive melanoma phenotypes. We observed the invasive phenotype melanoma cell cultures had modest decrease of 5% in global methylation compared to the proliferative phenotype melanoma cell cultures. This may be due to decreased DNMT1 protein expression in the invasive phenotype melanoma cells. Global methylation levels have been observed to decrease as a cancerous lesion progresses from a benign tumor to metastasis [22, 40], and we observed our invasive phenotype melanoma cell cultures had decreased DNA methylation levels and were more invasive than the proliferative phenotype melanoma cell cultures, suggesting the invasive cell cultures have progressed further in malignancy. Differential expression of the *de novo* DNA methyltransferase DNMT3b was also seen between the proliferative and invasive phenotype. These data are consistent with a model in which DNMT1 and DNMT3b have phenotype specificity and contribute to transcriptional heterogeneity by altering the methylation landscape of a melanoma cell in the context of melanoma phenotype switching. Pathway analysis of the 73 gene signature from the DNA methylation and gene expression array lead to the discovery of many transcription networks involved in development. These transcription factors were found to be hypomethylated and highly expressed in the invasive phenotype, which would suggest the invasive melanoma cell cultures may revert to a dedifferentiated state.

A number of other studies that have looked at genome-wide DNA methylation in melanoma have indicated that several tumor suppressors are silenced by DNA methylation compared to normal melanocytes [41-43] and compared to benign nevi [44]. Also, a recent study investigating 5-hydroxymethylation (5-hmC) in melanoma found a global decrease of 5-hmC was necessary for melanoma formation [45]. The results from these studies indicate aberrant DNA methylation is an important process in melanoma development and progression. In our work, we looked at the differences in DNA methylation landscape between ten primary melanoma cell cultures and uncovered two distinct populations, as previously demonstrated by gene expression microarray analysis from our group [13]. Surprisingly, the targets we found to be differentially methylated between the two phenotypes do not overlap with the targets found to be differentially methylated between normal melanocytes and melanoma, and benign nevi and melanoma. We did not detect any differential methylation in validated methylation gene sets such as *COL1A2, NPM2, HSPB6, DDIT4L, and MTIG* from Koga et al. [42] or *UCHL1, COL1A2, THBS1, and TNFRSF10D* from Bonazzi et al. [41]. As those studies were comparing the methylation state of normal melanocytes to melanoma and in this study we compare within melanoma phenotypes, this might indicate that a different set of pathways are activated or silenced by DNA methylation in melanoma progression compared to melanoma initiation. In either case, it is clear that epigenetic modifications such as DNA methylation play an important role in melanoma initiation as well as progression, and embryonic developmental program reactivation may be one of the critical outcomes of this modulatory activity.

In our study, a subset of our melanoma cell cultures had lower SOX9 expression due to a hypermethylated promoter and the other subset with high SOX9 expression had a hypomethylated promoter. We confirmed that SOX9 is regulated by DNA methylation by treating low SOX9 expressing cells with 5-aza-2-deoxycytidine treatment and saw reexpression of SOX9. To determine if the regulation of SOX9 by DNA methylation is a common mechanism in melanoma or just seen within our melanoma cell cultures, we interrogated the melanoma TCGA dataset for SOX9 and found that SOX9 gene expression and DNA methylation are anti-correlated at three consecutive methylation probes in 293 samples. This provides strong evidence that specific DNA methylation is the molecular mechanism that regulates SOX9 expression in melanoma. Alcazar et al. demonstrated that after Decitabine treatment of A375 and B16 melanoma cells, the promoter of SOX9 becomes hypomethylated and SOX9 is re-expressed with induction of p27 and p21 for cell cycle arrest [27]. Passeron et al. also observed that SOX9 was down-regulated in some melanoma cell lines and induction of SOX9 expression

in these melanoma cell lines resulted in lower proliferation due to upregulation of p21 [29]. We also overexpressed SOX9 in low SOX9 melanoma cell cultures and observed G1/S cell arrest, which is consistent with the study from Passeron et al. Although the proliferation rate is reduced, the invasive capacity of these SOX9 overexpressing cells is increased, which phenocopies the endogenous SOX9 expressing cells. Conversely, knockdown of SOX9 in the invasive phenotype melanoma cells reduced the invasive capacity of the cells. Microarray analysis of SOX9 overexpression revealed an EMT-like transcriptional signature and had 10% overlap with invasive phenotype gene signature which supports the notion that SOX9 is a factor that contributes to the invasive phenotype. *In vivo,* sox9 expression in B16F1 cells increases their metastatic potential causing more tumor lung nodules in the tail vein injection assay. Taken together, SOX9 is a gene that is regulated by DNA methylation and functionally, SOX9 mediates cell cycle progression, invasion and metastasis in melanoma.

The Cancer Genome Atlas (TCGA) is a great resource for clinical and next-generation sequencing data on human tumors. We took advantage of the melanoma dataset and demonstrated that *SOX9* expression levels have a significant impact on survival of metastatic melanoma patients but *SOX9* did not have a significant impact on survival in patients with primary melanomas. This could suggest that *SOX9* is required for progression of primary melanoma into metastasis and metastatic tumors with high SOX9 are more aggressive to the patient. There were no clinical metrics that could distinguish SOX9 high or low in primary melanoma. Only T1 and N0 stage in metastatic melanomas were significant between SOX9 high and low. Survival analysis of metastatic patients with high SOX9 expression versus low SOX9 expression revealed a significant difference in the overall 10 year survival rates. Patients with high-SOX9 expressing tumors had a 2.3 times increased risk of death compared to patients with low SOX9 expressing tumors. Based on these findings and the invasive properties of high SOX9 expressing melanomas, it would suggest that SOX9 expression in melanomas could push the tumor toward more aggressive metastasis. Thus, SOX9 could potentially be a prognostic marker for metastatic melanoma.

We performed differential gene expression analysis on the RNAseq dataset where we defined the SOX9 high group as having a minimum of 3 fold greater expression than the SOX9 low group. We only saw an overlap of 31 genes between both datasets however the overlap was significant as determined by hypergeometric distribution. The contribution of heterogeneity in the melanoma TCGA patient population would be one of largest factors for the difference in

gene signatures between our SOX9 microarray and TCGA RNAseq data. Nonetheless, the significant overlap of genes narrow down the potential targets of SOX9. To confirm that the targets of SOX9 have prognostic value for the patients, we performed survival analysis on all 31 genes. Surprisingly, 58% (11/19) of the genes down-regulated by SOX9 were associated with improved survival, which strongly suggests SOX9 represses a set of genes that decrease tumor malignancy. Genes that were up-regulated by SOX9 expression had little impact on patient survival which implies that SOX9 expression alone is sufficient to drive disease progression. From this list of 31 genes, 19 of them had a potential SOX9 binding site in its promoter. We could validate TMEM158, TBX3 and FYB as direct targets of SOX9 binding by chromatin immunoprecipitation. FYB is downregulated when SOX9 levels are high suggesting a repressive effect of SOX9 on this gene. FYB is required for inflammatory cytokine production [46] but no known link has been established with melanoma. TMEM158 is upregulated by SOX9 but no clear role has been established for the gene in melanoma. TBX3 is also upregulated by SOX9 and TBX3 is known to cause increased invasiveness in melanoma [47, 48], suggesting TBX3 could be an effector gene that drives the invasive phenotype we see in SOX9 high cells and in patients.

In conclusion, we found SOX9 to be regulated by DNA methylation, and high SOX9 expression leads to poor survival in melanoma patients due to the activation of EMT-like genes and the down regulation of potential tumor suppressor genes in melanoma cells. This was confirmed in vivo, and new direct targets of SOX9 that may mediate its function in tumor progression were identified by transcriptional profiling and chromatin-immunoprecipitation. Future therapies targeting SOX9 could be beneficial for patients to prevent progression and especially when combined with therapies targeting cells of the proliferative phenotype. Further investigation would be required to determine if SOX9 would have early prognostic value for tumor malignancy.

Acknowledgements

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genomic data. Finally, all research on human materials was done according to Swiss law and following the Declaration of Helsinki on medical research. Consenting patients contributed surplus melanoma biopsy material according to IRB approval numbers (Ek.647/800), and cell lines were generated following IRB KEK-ZH-Nr.2014-0425).

Materials & Methods

Cell culture. Melanoma cell cultures were established from surplus material from primary cutaneous melanoma and melanoma metastases removed by surgery [49]. Written informed consent was approved by the local IRB (EK647 and EK800). Clinical diagnosis was confirmed by histology and immunohistochemistry. Melanoma cells were released from tissue biopsies and grown as previously described [50]. Melanoma cell cultures were maintained in RPMI (Invitrogen, Carlsbad, CA, USA) supplemented with 5 mM glutamine, 1 mM sodium pyruvate and 10% heat-inactivated fetal calf serum, and cultured at 37°C and 5% CO_2. As RNA was extract previously from these cell cultures for gene expression array analysis, all cell cultures used for experiments in this paper were within 5 passages of the RNA isolation time point.

5-methylcytosine relative content analyses. Global DNA methylation level was evaluated by MethylFlash Methylated DNA Quantification Kit (Epigentek, Farmingdale, NY, USA) as per manufacturer's instructions.

MeDIP assay and analysis. The MeDIP assay was performed as described [31]. A monoclonal antibody to 5-Methylcytidine (BI-MECY-100, Eurogentec, Belgium) was used for immunoprecipitation. The immunoprecipated DNA and sonicated input DNA were differentially labeled with fluorescent dyes (Cy3 and Cy5, respectively) and hybridized to Human DNA Methylation 3x720K CpG Island Plus RefSeq Promoter Arrays (Roche Nimblegen, Madison, WI, USA). Acquisition and analysis was performed using Nimblescan 2.5 and R package DMR provided by Nimblegen. All data has been deposited into NCBI GEO GSE57971.

Gene expression analysis. Gene expression datasets were obtained from NCBI GEO GSE33728 [13], and analysis was performed by R using the limma package. P-values were adjusted by FDR multiple hypothesis test correction.

Bisulphite sequencing. Genomic DNA was extracted from primary melanoma cell cultures and subjected to bisulfite (BS) modification (EZ DNA Methylation Gold Kit, Zymo Research, Irvine, CA, USA). Primers used for bisulphite PCR are described below. To validate the DNA methylation status of individual DNA molecules, we cloned bisulfite-converted PCR fragments into the pCR2.1 vector using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA). Individual colonies were screened for the insert, and the region of interest was sequenced using M13 primers. A minimum of 5 clones were sequenced for each region of interest. Lollipop diagrams were generated using BiQ Analyzer [51].

Primers for bisulphite sequencing

Gene	Primer	Tm
SOX9_1	F: 5'-GGATTGGGGTTTTTTATTTTT-3'	59ºC
	R: 5'-TTCAATTTTCTTCCCTTTCCT-3'	
SOX9_2	F: 5'-AGGTTATTAGGGTAGATTGGAGG-3'	59ºC
	R: 5'5AAATACATATCCCATCACAACC-3'	

Treatment with Decitabine. Decitabine (5-Aza-2′-deoxycytidine, Sigma Chemical [Aza]) was dissolved in DMSO as a 10 mM stock solution, aliquoted and kept at −20°C. Primary melanoma cell cultures were seeded in Petri dishes (~5000 cells/cm^2) in RPMI untreated or treated with Aza (5 μM) for 72 hours, with fresh drug-supplemented medium every 24 hours.

mRNA expression analysis. Total RNA was isolated using Trizol according to manufacturer's instructions (Invitrogen). 1 μg aliquots of RNA were reverse transcribed with Reverse Transcription System (Promega) according to the manufacturer's instructions. Data collection and analysis were performed by ABI Viia7 Fast Real-Time PCR Systems (Applied Biosystems). Gene expression values of averaged triplicate reactions were normalized to *RPL28* expression levels. RPL28 primers are as follows: 5'-GCAATTGGTTCCGCTACAAC-3' and 5'-TGTTCTTGCGGATCATGTGT-3'.The primers for RT-PCR were purchased from QIAGEN: SOX9 (Hs_SOX9_1_SG)

Western Blot. Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed at 4ºC in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA), 137 mM NaCl, 10% glycerol, and protease and phosphatase inhibitors (Roche, Basel, Switzerland). Proteins were separated by SDS-PAGE using the NuPAGE SDS-

PAGE Gel System (Invitrogen) under reducing conditions. 15 µg of protein was mixed with 9 µl of NuPage LDS sample buffer (4x) (Invitrogen, NP0007), 3.6 µl of NUPAGE Sample Reducing (Invitrogen, NP0009) and filled up to 36 µl with RIPA buffer. This mixture was incubated at 85°C for 10 minutes while shaking at 900 rpm. Samples were loaded on NuPage precast gels (Invitrogen). Membranes were probed with the following antibodies: SOX9 (GTX109661, GeneTex, Hsinchu City, Taiwan); DNMT1 (ab13537, Abcam, Cambridge, UK); DNMT3a (ab2850, Abcam, Cambridge, UK); DNMT3b (ab16049, Abcam, Cambridge, UK); GAPDH (ab9483, Abcam, Cambridge, UK);

siRNA knockdown. Silencing RNA (siRNA) transfection of melanoma cells was carried out using INTERFERin transfection solution according to the manufacturer's protocol (Polyplus-transfection, Illkirch, France). Cells were transfected with 5 nM of siRNA (Qiagen) for 72 hours before RNA or protein was extracted. As control siRNA, the All-Star negative siRNA sequence (Qiagen) was used, and gene-specific siRNAs targeting siSOX9 (SI00007595, SI00007609) were obtained from Qiagen.

SOX9 lentiviral transfection

Lentiviral particles containing plasmids expressing full-length SOX9 cDNA or eGFP were transfected into melanoma cells for 48 hours. Media supplemented with 4 ng/mL blasticidin was used for selection. After 1 week of selection, protein lysate was extracted and analyzed for SOX9 expression. Plasmids for eGFP and SOX9 were a kind gift from Dr. Thierry Passeron [29].

SOX9 transient transfection

Empty vector or vector containing murine sox9 (a kind gift from Prof. Lukas Sommer) was transfected into B16F1 cells with jetPEI (Polyplus, France) according to manufacturer's protocol. Protein was isolated at 24 hours, 72 hours, 144 hours and 288 hours after transfection and analyzed for sox9 expression by western blot.

Microarray and pathway analysis

Gene expression of eGFP and SOX9 transfected cells were analyzed using the Affymetrix Human Genome U133 Plus 2.0 Array at the Functional Genomics Center Zurich (FGCZ). Differential gene expression was determined by R package limma [52]. Pathway analysis was performed using MetaCore (GeneGo Inc, New York, USA).

Proliferation and Cell cycle analysis

For cell cycle analysis, the Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Invitrogen) was used. Cells were labelled with PI according to the manufacturer's protocol and the DNA content was measured using a BD FACSCanto II flow cytometer (BD Biosciences) and BD FACSDiva software (BD Biosciences). Cell cycle analysis was performed in triplicate.

Boyden chamber invasion assay. Cells were seeded on FluoroBlok 24-multiwell Insert System (351157, BD Biosciences) and Biocoat Tumor Invasion System (354165, BD Biosciences). The invasion assay was performed as per manufacturer's instructions. Migrated and invaded cells were labeled with Calcein AM fluorescent dye (354216, BD Biosciences) and fluorescence was measured with Tecan GENios (Tecan, Männendorf, Switzerland) using 485 nm excitation and 535 nm emission. Relative invasion was calculated as the ratio of the fluorescence of invading cells of the Biocoat Tumor Invasion System divided by the fluorescence of migrating cells of the FluoroBlok 24-multiwell Insert System. Boyden chamber assays were performed in triplicate.

Viability assay

Cells were seeded in 24-well microplates at a density of 2×10^4 cells, and cell growth was determined with a standard colorimetric assay measuring 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO, USA) reactivity after 72 hours. MTT assays were performed in triplicate.

Chromatin Immunoprecipitation

ChIP analysis was performed as previously described [53]. The Sox9 antibody was from Santa Cruz Biotechnology (sc-20095, Santa Cruz Biotechnology). Primer sequences were designed around SOX9 binding motifs [54, 55] from the transcription start site (TSS) to 3 KB upstream of the TSS. Primers for p21, COL2A1, TMEM158, TBX3, FYB, SOX10 and IP10 are as follows:

COL2A1-F	ATCCTCCTTTGTGAGGCTTGTT
COL2A1-R	AGTACGAGAGAACCCACTGGAC
p21-F	TGATGTGCCACAGTTCACAA
p21-R	TCCTGCCAGTTTTCCTGTTC
TMEM158-F	TCTGCTGTGTTGGAGCCATT
TMEM158-R	GTCTCGCCTTAGTGCTACCG
TBX3-F	CTCGCCCCTTTCTTTCCCTT
TBX3-R	GGGGGTGTTATGAGCCAACA

FYB-F	CTCACATTGCATGGGGACG
FYB-R	ATGGGCTTATCACCGGAAGG
SOX10-F	CCTCTGCCTCGTGTGACTAC
SOX10-R	TCCTGTCTGGAGTGGGCTG-
IP10-F	GGGAAATTCCGTAACTTGGA
IP10-R	AAGCCATTTTCCCTCCCTAA

In vivo metastasis

B16F1 cells were transfected with empty vector or vector containing murine sox9. 2 x 10^5 cells were injected intravenously into C57BL/6J mice, 5 mice per group (Harlan Laboratories). After 12 days mice were sacrificed and lungs were examined for metastasis. Statistics were performed using the Mann-Whitney U test. All animal experiments have been approved by the veterinary authorities of Canton of Zurich, Switzerland, and were performed in accordance with Swiss law.

TCGA analysis

The SKCM DNA methylation, RNA-seq and clinical dataset were downloaded on July 28, 2014 for analysis (http://cancergenome.nih.gov/). Normalized reads from the level 3 RNA-seq data was used for analysis. The dataset was segregated into primary tumors and metastatic tumors for analysis. Chi-squared test was performed on the clinical parameters between the SOX9 high and low groups. Differential expression was analyzed with voom from the limma package [52]. Log rank test and Cox proportional hazard ratio were analyzed by the survival R package [56]. DNA methylation β-values were calculated by minfi [57]. Correlation was calculated by Spearman's rank correlation coefficient.

Conflict of interest

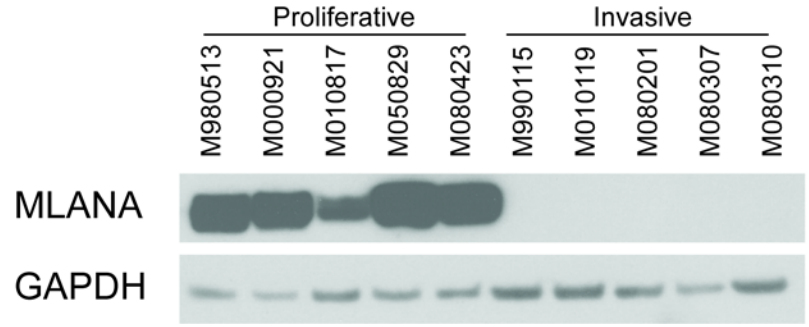
The authors disclose no potential conflicts of interest.

Authors' Contribution

PFC, OS, DSW, DZ, SCF, BB, MMIR, OME, and SMG performed the experiments. PFC, OS, DSW, DZ, and SCF analyzed the data. PFC, KSH, RS, RD and MPL contributed to the overall design of the study and directed research. PFC, SH, LS, RD and MPL drafted the manuscript. All authors read and approved the final manuscript.

Supporting Information

Supplemental Figure 1



Supplemental Figure 1. MLANA expression between the proliferative and invasion phenotype. MLANA expression is a marker for the proliferative phenotype. Ten melanoma cell cultures were divided into the proliferative and invasive phenotype by MLANA. GAPDH was used as the housekeeping gene.

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8. Discussion

Melanoma is a highly heterogeneous disease as seen by the multiple unique clinical presentations, the multiple genetic aberrations it can harbor, and the plasticity in behavior demonstrated by phenotype switching. Intratumoral heterogeneity and intrapatient heterogeneity have created a multitude of challenges for therapies that could be applied to all melanoma patients (1, 2). The first successful melanoma therapeutic has been $BRAF^{V600E}$ inhibitor therapy but that only targets 40% of melanoma patients and most patients eventually relapse (3). To combat the relapse to targeted therapy, combination of $BRAF^{V600E}$ and MEK inhibitors have seen higher responses but only extend the progression free survival about 4 months (4). Another therapeutic for melanoma is anti-CTLA4 immunotherapy. It can be administered to all melanoma patients but it only has a 10% response rate (5). Improved immunotherapy has been developed recently and response rates have improved to 40% with anti-PD1 immunotherapy (6). The complexity of treating melanoma patients is due to the diverse array of acquired and intrinsic resistance mechanisms to therapy which present huge challenges in generating long term remissions in patients (7, 8). Although great strides have been made in deciphering melanoma heterogeneity and uncovering resistance mechanisms to therapy, there still remains much to be discovered. It seems targeting one subpopulation is not enough for effective therapy, thus, characterization of melanoma subpopulations, and in particularly the signaling events that regulate phenotypic heterogeneity, might lead to the development of combination therapies targeting multiple subpopulations of tumor cells.

Phenotypic heterogeneity in an individual melanoma tumor could arise from a variety of mechanisms including, epigenetic or genetic mutations, micro-environmental pressures, and reversible gene expression patterns. Our group has proposed phenotype switching as a mechanism for melanoma heterogeneity (9-11). The phenotype switching model defines two subpopulations of melanoma cells, the proliferative phenotype and the invasive phenotype. The proliferative phenotype is defined by expression of melanocytic markers like MITF, SOX10, and TYR and is characterized by a high proliferation rate and low invasion rate. The invasive phenotype is defined by expression of mesenchymal markers like WNT5A, TGFB, and SOX9 and is characterized by a low proliferation rate and high invasion rate. In Chapter 5, we applied our phenotype gene signatures across all available melanoma microarray data in the GEO database. We first used a training set of 192 melanoma cell cultures and lines to define the 97 gene signature that characterizes the proliferative and invasive phenotype. We tested this gene

signature on a test set of 318 melanoma cell cultures and lines and 81% of the samples have correlation to the proliferative or invasive gene signature. The two phenotypes are independent of mutation status, which suggests these phenotypes are driven by cell signaling. We also validate the phenotype of a few external melanoma cell cultures with *in vitro* experiments for proliferation and invasion. The results from this study demonstrate that melanoma cell phenotype is dictated by gene expression irrespective of genetic background.

One gene of interest from the proliferative gene signature is SOX10. SOX10 is one of the essential genes involved in neural crest specification, differentiation and survival (12). SOX10 is critical for NCSC self-renewal and survival (13) and SOX10 mutation in mice causes a *Dominant megacolon (Dom)* phenotype (14). The *Dom* mice are characterized with aganglionic megacolon, deafness and hypopigmentation. In humans, SOX10 mutations cause Waardenburg Hirschsprung syndrome which share the same phenotype as the *Dom* mice (15). The hypopigmentation defect was the first indication that SOX10 plays a role in dictating the melanocytic lineage in NCSCs. SOX10 was later found to bind and activate the promoter of MITF, the master regulator of melanocytes (16). MITF is a transcription factor essential for melanocyte function, which activates a wide array of genes involved in cell-cycle progression (i.e. CDK2, p16) and melanocyte differentiation (i.e. TYR, MLANA) (reviewed in (17)). MITF was found to be amplified in 10-20% of melanoma (18) and expression of MITF was highly variable across melanoma specimens with the majority of primary melanomas having high MITF expression and a large percentage of metastatic melanomas with low MITF expression (19). SOX10 is also essential for normal melanocyte function and melanoma formation (20-23). In the *Tyr::Nras*^{Q61K} Ink4a$^{-/-}$ mouse model, melanoma tumors form spontaneously and are reflective of human melanoma, where 20% of patients have an NRAS mutation and often have p16 silenced (24). SOX10 haploinsufficiency in the *Tyr::Nras*^{Q61K} Ink4a$^{-/-}$ mice rescues the mice from developing melanoma tumors and the mice have a normal pigmentation pattern. This suggests that in an oncogenic environment, lower levels of SOX10 prevents melanoma initiation and still allows for normal melanocyte function. High levels of SOX10 were found to be a poor prognostic marker for melanoma patients (25, 26). These findings highlight the importance of cell lineage developmental pathways in oncogenesis and perhaps targeting SOX10 in melanoma could be a potential therapeutic target.

From the microarray data in Shakhova et al. shRNA knockdown of SOX10 elevates SOX9 expression (22). SOX9, like SOX10, belongs in the SOXE family of transcription factors and can play redundant roles in the neural crest (27, 28). The primary role of SOX9 is in gonad and

chondrocyte development and loss of SOX9 does not affect the melanocytic lineage (29-31). Interestingly, ectopic expression of SOX9 under the DCT promoter affects melanoblast development, where the mouse has light coat pigmentation, dorsal white spotting and microphthalmia (32). Taken together, this could suggest that SOX9 has an antagonistic role to SOX10 in melanocyte and melanoma function. In Chapter 6, we investigated the role of SOX9 on SOX10 in the context of melanoma formation. SOX9 and SOX10 mRNA and protein levels had an anti-correlative relationship in melanocytes, naevi, and melanoma and SOX9 binds to the promoter of SOX10. Overexpression of SOX9 decreased SOX10 levels and caused the melanoma cells to enter G1 cell cycle arrest. Using *Tyr::Nras*^{Q61K} *Sox10*$^{fl/-}$ and *Tyr::Nras*^{Q61K} *Sox10*$^{fl/fl}$ mice, homozygous deletion of SOX9 restored the hyperpigmentation phenotype as originally seen in *Tyr::Nras*^{Q61K} mice, suggesting that the effects of SOX10 loss are due to SOX9 expression. Finally, looking at The Cancer Genome Atlas (TCGA) melanoma database, patients with high levels of SOX10 had poor prognosis compared to patients with low SOX10. These results indicate the importance of SOX9 in melanoma initiation suggesting it could play a potential tumor suppressor role by inhibiting SOX10. In terms of phenotype switching, the antagonistic role of SOX10 and SOX9 would fit the model, where SOX10 would be part of the proliferative phenotype and SOX9 would be part of the invasive phenotype. High SOX10 levels in mice caused increased proliferation of melanocytes which led to melanoma formation and suppression of SOX10 by SOX9 reduced tumor burden. The phenotype switching model predicts that high SOX9 levels would lead to increased invasion, but this was not addressed in this study. We investigate the invasive role of SOX9 in melanoma in Chapter 7.

In addition to reversible gene expression as a contributor to melanoma heterogeneity, epigenetic mechanisms are also reversible and heritable. Thus epigenetic mechanisms can provide a stable mechanism for a cell to maintain a certain phenotype but also allow the cell to transition to another phenotype. DNA methylation is an epigenetic mechanism that is aberrantly expressed in cancer (33). As the genes in the proliferative and invasive phenotype are stably expressed, it is plausible that some of the genes are regulated by DNA methylation. To date, more than 70 genes have been found to be hyper-methylated in melanoma compared to normal melanocytes (reviewed in (34)). These genes encompass several important signaling pathways including MAPK, PI3K, and WNT and pathways involving cell cycle, apoptosis, and DNA repair. The majority of studies investigating the landscape of DNA methylation in melanoma compare melanoma to normal skin or to benign nevi (35-38). In Chapter 7, we investigate the DNA methylation landscape within melanoma, comparing the proliferative phenotype to the invasive

phenotype. Taking five proliferative phenotype cell cultures and five invasive phenotype cell cultures, we compared the DNA methylation profile and gene expression profile and found a number of genes that had anti-correlative DNA methylation and gene expression between the two phenotypes. One of the genes validated was SOX9. From Chapter 6, we know that overexpression of SOX9 suppresses the expression of SOX10 and causes the cells to enter G1 cell cycle arrest. Invasive phenotype melanoma cell that endogenously express SOX9 have low levels of SOX10 and have a decreased proliferation capacity but are not arrested. It could be an artifact of overexpression that causes the cells to enter cell cycle arrest. The role of SOX9 in invasion was addressed with *in vitro* Boyden chamber assays on SOX9 knockdown and SOX9 overexpression and *in vivo* metastasis assays with SOX9 overexpression in B16F1 cells. SOX9 knockdown decreased the invasive capacity of the melanoma cells and SOX9 overexpression increased the invasive capacity of the melanoma cells. *In vivo*, B16F1 cells are not known to metastasize to the lung in a tail vein injection assay, but with SOX9 overexpression, melanoma nodules were detected in the lung. We also addressed the role of SOX9 in survival and discovered that patients with high SOX9 expression have poor prognosis compared to patients with low SOX9 expression in the TCGA melanoma dataset. Taken together, this study demonstrates that SOX9 is regulated by DNA methylation and its role in melanoma is to induce melanoma cell invasion which leads to poor prognosis for the patient. This would suggest that targeting the invasive phenotype cells perhaps through SOX9 would be beneficial for the patient.

Interestingly, invasive phenotype cells are more resistant to targeted inhibitor therapy, suggesting it as an intrinsic resistance mechanism. Studies from Konieczkowski et al. and Zipser et al. both demonstrated that melanoma cells with low MITF expression were less sensitive to BRAF and MEK inhibitors and these drug resistant melanoma cells expressed high levels of NF-kB, AXL, WNT5A and TGFB (39, 40). These findings have potential implications for patient stratification in therapy treatment, as patients could be pre-screened for these invasive marker genes to determine the benefit of a targeted therapy. One possible avenue to combat intrinsic resistance is to target the invasive phenotype. Indeed a recent study demonstrated that methotrexate could push invasive phenotype cells toward the proliferative phenotype, thus sensitizing them to a TYR-processed antifolate prodrug (41). The ineffective results from single inhibitor treatment indicate that melanoma heterogeneity should be addressed when considering therapeutic targets, which suggests targeting multiple subpopulations might be more beneficial for the patient. Indeed, the current trend for melanoma therapy is combination

therapy; current strategies include inhibition of multiple targets of the same pathway, i.e. BRAF and MEK inhibitor combination treatment, or inhibition of parallel pathways, i.e. BRAF and PI3K inhibitor combination treatment (42, 43), but inhibitors targeting phenotype switching have not been introduced in human clinical trials yet.

In this thesis, we suggest that targeting SOX10 and SOX9 could be beneficial for patient survival, as high levels of SOX10 expression are required for melanoma formation and high levels of SOX9 expression are required for melanoma invasion. Survival analysis of patients with high expression of SOX10 or high expression of SOX9 both have poorer prognosis. This indicates that either phenotype for the patient is detrimental to their survival. The proliferative phenotype could cause increased tumor size and the invasive phenotype could cause more metastasis. We show the dynamic nature of phenotype switching from the antagonistic relationship of SOX10 and SOX9 (Figure 1). Expression of SOX9 causes down regulation of SOX10 and vice versa. It would suggest that only targeting one phenotype leads to the expression of the other. For effective therapy, we would propose targeting both phenotypes. Transcription factors are difficult targets for current therapeutics thus elucidating downstream effector molecules for SOX10 and SOX9 would be crucial for drug design. The promising results from Saez-Ayala M et al. on combination therapy targeting both the proliferative and invasive phenotype with the TYR-processed antifolate prodrug and methotrexate in mice provide an encouraging rationale for developing more drugs that inhibit both phenotypes. Another potential target for therapy is targeting phenotype switching itself. Since the DNA methylation landscape is unique for the proliferative and invasive phenotype, utilizing a DNMT inhibitor like Azacytidine in combination with current therapies which could prevent the melanoma cells from switching phenotypes and developing resistance.

In conclusion, the phenotype switching model provides a model for melanoma progression where melanoma cells transition between states of proliferation and invasion. In Chapter 5, we provided evidence that these phenotypes exist within melanoma cell cultures used worldwide. In Chapter 6, we demonstrate that SOX9 is a negative regulator of SOX10 in melanocytes and melanoma. In Chapter 7, we demonstrate that SOX9 is regulated by DNA methylation and is a negative prognostic factor for melanoma patients. All these data contribute to a growing field of phenotype switching in melanoma.

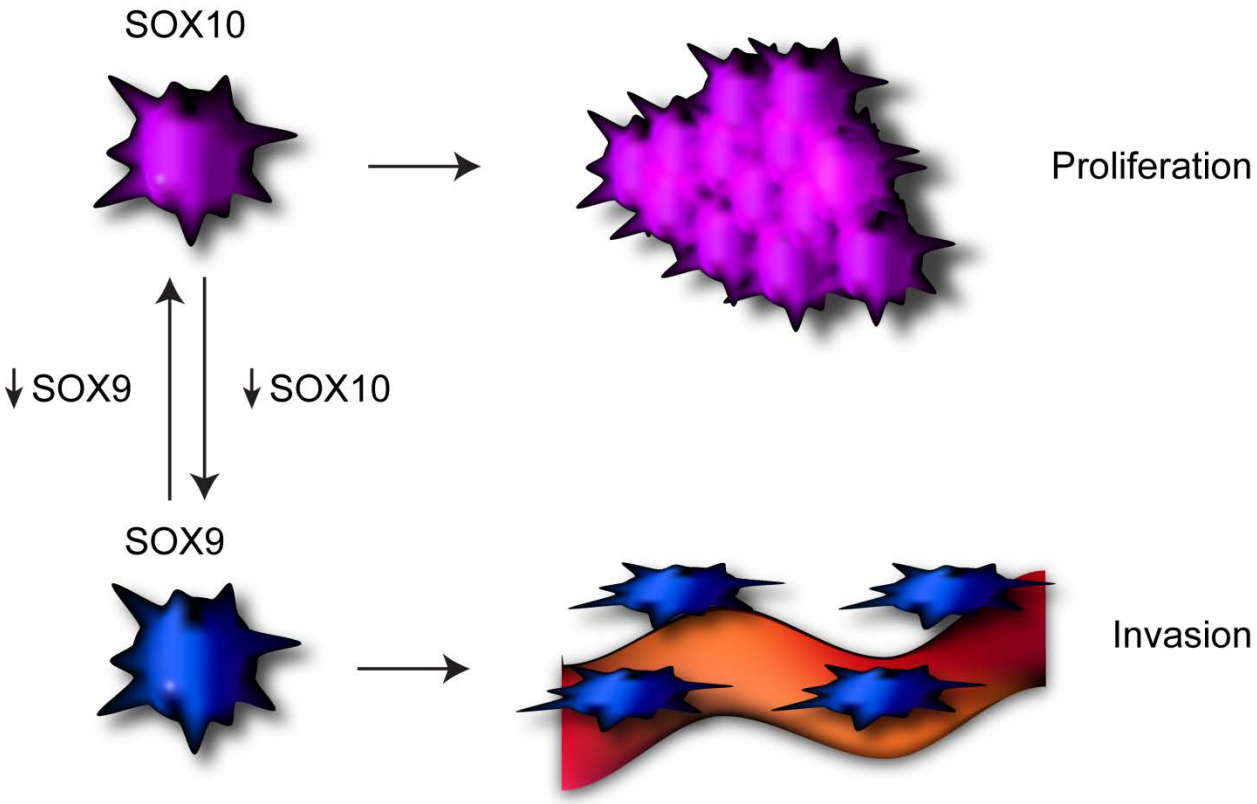

Figure 1. Phenotype Switching Model with SOX10 and SOX9. SOX10 expressing cells are proliferative and cause growth of the tumor. Downregulation of SOX10 leads to expression of SOX9. SOX9 expressing cells are invasive and cause tumor invasion. Downregulation of SOX9 leads to expression of SOX10. Melanoma tumors are comprised of both SOX10 and SOX9 populations thus leading to growth and invasion of the tumors.

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